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Studies on Deoxyribonucleases.

by Peter John Curtis, B. Sc.

A nuclease from rat liver has been identified which shows a preference for denatured rather than native DNA as its substrate. It was considered necessary to purify this enzyme before undertaking studies on its properties and specificity. A suitable source of the liver nuclease was shown to be mitochondria. The enzyme was released into solution from isolated mitochondria by treatment with a solution of triton-X-100. The enzyme solution was fractionated firstly with saturated ammonium sulphate taking the precipitate obtained between 35-55% saturation, followed by acetone treatment where the precipitate obtained between 36.9-46.9% acetone concentration was collected. Further purification was achieved by the use of column chromatography. Passage through a column of diethylaminoethyl-cellulose equilibrated with 0.01M tris-HCl buffer pH 8.0 resulted in the removal of 95% of the protein though the majority of the enzyme was not adsorbed. Gel filtration on a column of Sephadex G-75 resulted in a further 4 fold increase in the specific activity.

The overall purification obtained was 715 fold. The purified liver nuclease showed negligible acid DNase, phosphatase

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and phosphodiesterase activities, though it hydrolysed both native DNA and RNA as well as denatured DNA. The activities towards these three substrates were eluted together from columns of Sephadex G-75 and G-200 suggesting that they belong to one and the same protein. The purified liver nuclease is rather unstable in that it loses its activities when stored in ice, frozen or freeze-dried. However when stored in the presence of 20% glycerol or 0.01M 2-mercaptoethanol in ice the DNase and RNase activities were retained for upto 2 weeks. Beyond this period both activities decreased, DNase more rapidly than RNase, suggesting that they belong to separate proteins.

The molecular weight of the liver nuclease was estimated to be about 44,000 from its elution profile on a column of Sephadex G-75. The properties of the two activities of the liver nuclease were examined. Both showed a sharp pH optimum about 6.8-7.0 and an absolute requirement for  $Mg^{2+}$  or  $Mn^{2+}$ . The optimal concentrations of  $Mg^{2+}$  and  $Mn^{2+}$  were similar and little difference was observed when DNA or RNA were used as substrates.  $Ca^{2+}$  showed no stimulatory effect and in the presence of the optimal concentration of  $Mg^{2+}$ ,  $Ca^{2+}$  inhibited both activities as did NaCl. The two activities were readily denatured by heating. In all these properties the activities towards denatured DNA and RNA are



very similar thus providing further support for the hypothesis that the two activities belong to one and the same protein.

The specificity of the nuclease towards DNA and RNA were studied. Both DNase and RNase exhibited an endonucleolytic mode of action since only 8% of the digest consisted of mononucleotides. Experiments were carried out to determine whether the enzyme split the phosphodiester bonds between the 3'-carbon and phosphorus or the 5'-carbon and phosphorus. Treatment of the oligonucleotides produced by the enzyme with snake venom and spleen phosphodiesterase showed that the oligodeoxyribonucleotides and oligoribonucleotides were terminated by 5'-phosphate groups. Finally, the enzyme showed little specificity towards the bases in DNA and RNA. This lack of specificity was demonstrated by determining the proportion of the bases at the 3' and 5' ends of the oligonucleotides produced by the digestion of the DNA and RNA by the enzyme. These results further illustrate the similarity of the two activities, which are endonucleases producing oligonucleotides terminated by 5'-phosphate groups.

**Studies on Deoxyribonucleases.**

by

Peter John Curtis B.Sc.

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## ABBREVIATIONS.

The following abbreviations will be used in this thesis.

DNA	Deoxyribonucleic acid or deoxyribonucleic acid.
RNA	Ribonucleic acid or ribonucleate.
sRNA	Soluble RNA.
Poly A, etc.	Polyribonucleotide strand containing only adenylate residues; similarly for the other synthetic polynucleotides.
DNase	Deoxyribonuclease.
RNase	Ribonuclease.
AMP, GMP, IMP, UMP,	5'-phosphates of ribosyl adenine, guanine,
UMP, rTMP.	hypoxanthine, <sup>uracil</sup> cytosine, thymine.
dAMP, etc.	5'-phosphates of 2-deoxyribosyl adenine, etc.
3'-AMP, etc.	3'-phosphates of ribosyl adenine, etc.
3'-dAMP, etc.	3'-phosphates of 2-deoxyribosyl adenine, etc.
Ado, Guo, Cyt, Urd,	Ribosyl adenine, guanine, cytosine, uracil.
Ino, Thd.	hypoxanthine, thymine.
dAdo, etc.	2-deoxyribosyl adenine, etc.
Pu, Py.	Denote purine, pyrimidine bases respectively.

p-Nitrophenyl-pT

p-Nitrophenyl deoxythymidine  
5'-phosphate.

p-Nitrophenyl-pU

p-Nitrophenyl uridine 5'-phosphate

Tp-nitrophenyl

p-Nitrophenyl deoxythymidine 3'-  
phosphate.

Oligonucleotides will be represented according to the system suggested by Heppel, Ortiz & Ochoa, (1957). Thus,

pX, pXpY, pXpYpZ

represent mono-, di-, and tri-  
nucleotides terminating in 5'-  
phosphate groups.

Xp. XpYp, XpYpZp

represent mono-, di- and tri-  
nucleotides terminating in 3'-  
phosphate groups.

dpX, etc. and dXp, etc. represent the corresponding deoxy-  
ribosyl compounds.

EDTA

Ethylenediaminetetra-acetic acid

Tris

Tris-(hydroxy-methyl)-amino methane

DEAE-

Diethylaminoethyl-.

CM-

Carboxymethyl-.



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CHAPTER I  
General Introduction.

Living organisms have two unique characteristics, the ability to grow and reproduce. Starting with simple molecules they can assemble these into macromolecules which are then arranged to form a living organism. This organism will be a replica of the original parent. The understanding of these processes has depended upon the discovery of the structure of deoxyribonucleic acid (DNA) in particular its secondary structure. Another characteristic of living organisms was adequately described 100 years ago by Darwin in his book "The Evolution of the Species". This is the ability of life to evolve from a single form to a variety of related forms. Darwin thus pointed out that the many thousands of distinct species of plants and animals did not arise by simultaneous creation, but by evolution from common ancestors. However it is important to realise that each species is a stable entity for many thousands of years. The continuation of the species can be partly interpreted in terms of the known metabolic stability of cellular DNA. Whereas evolution depends upon a ability for changes to occur at a very slow rate so that any mutation has an opportunity to show its value. And DNA can undergo changes in its structure under the influence of various agents. But there is an apparent paradox here; the continuation of a species depends on the metabolic stability of the DNA molecule, but many kinds of animal, bacterial and



plant cells contain a variety of enzymes capable of hydrolysis of the DNA. This paradox has not yet been adequately answered.

# 1. The structure of the nucleic acids

## 1.1. Deoxyribonucleic acid.

The structure of DNA is so well established that only a brief summary will be given. DNA contains three basic components, phosphoric acid, 2-deoxy-D-ribose and an organic base. There are normally only four bases found in DNA, adenine, guanine, cytosine and thymine. Certain other bases occur in small amounts in particular cells. These bases include 5-methylcytosine, which was first discovered in calf thymus DNA and later in DNA from mammalian, fish and insect sources, and in larger amounts in wheat germ DNA (Wyatt, 1951); 5-hydroxymethylcytosine in the T-even bacteriophage where it replaces cytosine (Wyatt and Cohn, 1952); 6-N-methylaminopurine in certain strains of Escherichia coli (Dunn and Smith, 1958).

DNA is a linear polymer whose basic unit is a 5'-nucleotide (fig.1.). The backbone consists of deoxyribose linked by a phosphate bridge between the 3'- and 5'-hydroxyl groups. The bases lie in a plane perpendicular to the chain axis. The sequence of the bases provides the genetic information. But the experimental determination of



Figure 1.

A section of the polynucleotide chain in the DNA molecule.

(From Davidson, J.N., "The Biochemistry of the Nucleic Acids", 4th Ed., Methuen & Co. Ltd., London, 1960).

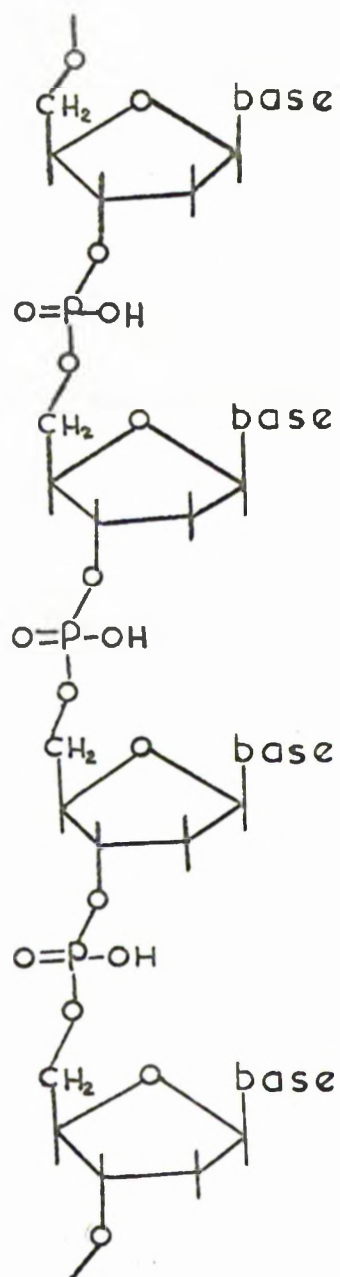


Figure 1.



the sequence of a DNA molecule remains too complex. However Chargaff (1950, 1951) showed that DNA from a variety of sources contained certain regularities in its base composition i.e. (i) adenine is equal to thymine (ii) guanine is equal to cytosine. The reason for this became clear when it was discovered that DNA existed as a relatively rigid rod consisting of two chains wound around a common axis to form a regular  $\alpha$ -helix. (Wilkins, Stokes & Wilson, 1953; Watson & Crick, 1953). This helix has a diameter of  $17\text{\AA}$ , a pitch of  $34\text{\AA}$ , with 10 bases per turn of the helix. The bases face inwards and lie perpendicular to the long axis. This brings the bases of the two chains close enough for hydrogen bonding, and it is this hydrogen bonding that holds the two chains together. Such a helix imposes sterical limitations on the bases that can form a hydrogen bonding pair. A purine-purine pair is too large, and pyrimidine-pyrimidine is too small. Only adenine-thymine and guanine-cytosine pairs fit without straining the helix. This accounts for Chargaff's findings (1950, 1951).

The double helix is not universal since the DNA from the bacteriophage  $\phi$ X 174 was found to be single stranded (Sinsheimer, 1959a, 1959b). The two strands can also be separated by rupture of the hydrogen bonds, a process termed denaturation. Denatured DNA behaves as a flexible, loosely-



coiled polyelectrolyte chain, very much dependent in its properties on the ionic environment, in contrast to the behaviour of double stranded DNA. The denatured DNA of viruses has a molecular weight one half of the double-stranded DNA, but the molecular weight of mammalian DNA remains constant on denaturation. This is probably due to the fact that mammalian DNA is very heterogeneous in composition, and the separated strands are therefore more likely to aggregate since the likelihood of two non-complementary strands finding short regions that can pair would be higher than for DNA from viral or bacterial sources (Marmur, Rownd & Schildkraut, 1961).

The DNA in the cell exists mainly in combination with basic protein molecules, and forms the microscopically visible chromosomes. But the relation of the DNA molecules within the macromolecular structure of the chromosomes is as yet unknown (Taylor, 1963; Thomas, 1963). The molecular weight of DNA isolated from chromosomes, by taking careful precautions to avoid breakdown, has been estimated to be about 5 - 10 million (Cavalieri, Rosenberg, & Deutsch, 1959; Davison, 1959; Zubay & Doty, 1959; Cavalieri, Finston & Rosenberg 1961). T2 and T4 bacteriophage DNAs have been isolated with a molecular weight of about  $130 \times 10^6$  (Levinthal & Davison, 1961), and Cairns (1962) has obtained electron micrographs of E. coli in a circular form and



and therefore presumably whole. If the thread that is shown is double stranded, then its length corresponds to a molecular weight of  $10^9$ .

The presence of significant amounts of extranuclear DNA occurring in the cytoplasm of the amphibian oocyte (Stich, 1962), the kinetoplasts (Clark & Wallace, 1960), and in chloroplasts of plant cells (Chun, Vaughan, and Rich, 1963) might be considered as an exception rather than as a rule, because in these cases DNA is associated with cellular components absent from animal cells. Recent findings, however, have indicated the presence of DNA in mitochondria of various mammalian tissues (Nass & Nass, 1963; Kalf, 1964) as well as in yeast (Schatz, Haslbrunner, & Tuppy, 1964) and Neurospora crassa (Luck & Reich, 1964). Since mitochondria are present in most cells, extranuclear DNA must be recognised to be more ubiquitous than had hitherto been accepted. The function of DNA within these various organelles is suggested by the existence of extrachromosomal genetic factors controlling mitochondrial function in yeast cells (Ephrussi & Hottinguer, 1951) and the occurrence of cytoplasmic inheritable factors controlling chloroplast function (review: Granick, 1963; Wilkie, 1964). On the basis of such evidence it has been suggested that these organelles, chloroplasts, kinetoplasts, and mitochondria are,



at least partially, autonomous self-replicating bodies, in which case the DNA would provide the instructions for their development. To substantiate this there are the findings of Luck & Reich (1964) of the presence of DNA-dependent RNA polymerase (E.C 2.7.7.6) in the mitochondria of N.crassa., and that of Kalf (1964) of a similar activity and in addition the ability to incorporate amino acids in lamb heart mitochondria.

### 1.2. Ribonucleic Acid.

In RNA, the pentose sugar D-ribose replaces 2-deoxy-D-ribose in forming with phosphate the backbone of the molecule as in DNA. The bases found in RNA are mainly adenine, guanine, cytosine and uracil. Several methylated bases have been identified in sRNA in small amounts, for example 5-methylcytosine, 6-methylaminopurine, 1-methylguanine, and 2-methylamino-6-hydroxypurine (Adler, Weissman, & Gutman, 1959; Dunn, Smith, & Spahr, 1960; Dunn, 1959, 1961). The nucleoside 5-ribosyluracil, or pseudouridine has also been identified as a minor constituent of sRNA (Cohn, 1960).

The RNA in a cell exists as two major components, ribosomal RNA which accounts for 80% of the total amount, and sRNA amounting to 15% of the total. sRNA has a molecular weight of approximately 25-30,000 (Tissieres, 1959). Evidence from X-ray studies has suggested that this molecule has a



double stranded helical structure (Spencer, Fuller, Wilkins & Brown, 1962). McCully & Cantoni (1962) have proposed that the helix is composed of the two halves of the chain hydrogen bonded together to form a hairpin structure. The two ends of the chain would then form the amino acid receptor site, and the loop would presumably contain the identifying triplet and the unusual bases that cannot fit into the double helix.

The precise secondary structure of ribosomal RNA has not been exactly determined. There are two sizes for E. coli; one has a sedimentation of 27s obtained from 50s ribosomal particle having a molecular weight of  $1.1 \times 10^6$ , and one with a sedimentation of 16s from 30s particle with a molecular weight of  $0.5 \times 10^6$ . Titration studies (Cox, Jones, Marsh & Peacocke, 1956) and X-ray data (Rich & Watson, 1954a, 1954b) indicated the existence of some elements of a Watson-Crick type of structure in a RNA solution. The helical structure appears to exist only in short regions made up by intrachain hydrogen bonding. Such regions may not be perfect helices, as it has been suggested (Fresco, Alberts & Doty, 1960) that unmatched bases within a helical region would be able to loop out. Doty, Boedtker, Fresco, Hall & Haselkorn (1959) and Doty, Boedtker, Fresco, Haselkorn & Litt (1959) and Boedtker (1959, 1960) have expressed the



opinion that RNA molecules in solution have no tertiary structure. However Spirin (1960) has proposed that there are certain regularities in the arrangement of helical sections in a RNA chain on the basis of experiments by Dvorkin & Spirin (1960). Whatever is the case, the secondary and tertiary structure of RNA in solution can hardly be considered as the original native structure equilibrium figure. However, studies on ribosomes by Hall & Doty (1959) showed hypochromicity and melting behaviour similar to free RNA. And x-ray studies (Zubay & Wilkins, 1960; Klug, Holmes & Finch, 1961) did not detect any significant difference between free RNA and RNA within ribosomes.

## 2. The Nucleases.

The term nuclease refers to a subgroup of phosphodiesterases (EC 3.1.4.1.) which attack the internucleotide bonds in the nucleic acids. Laskowski (1961) suggested that four criteria are used to classify the nucleases. They are:-

- (i) Susceptible substrate, ie. RNA, DNA or both.
- (ii) Products of hydrolysis, ie. either 3'-phosphate or 5'-phosphate terminated nucleotides; there appear to be no intermediate types.
- (iii) Type of attack, ie. either exonucleolytic or



endonucleolytic. Exonuclease activity refers to hydrolysis of the polynucleotide from one end producing mononucleotides only; they are commonly termed phosphodiesterases. Endonuclease activity is that attacking internucleotide bonds within the molecule. (fig. 2).

- (iv) Preferential linkage, i.e. whether the enzyme shows specificity toward either of the bases on each side of the bond that is hydrolysed.

A complete classification based on these four criteria must be however a tentative one, for few nucleases have been clearly defined in these four criteria.

Such classification is of limited value since it will group together enzymes obtained from bacterial, plant and animal sources. Such enzymes grouped together on the basis of the above classification cannot then be assumed to be similar with respect to amino acid composition, secondary and tertiary structure of the protein, and perhaps most important, function. This property would provide a more meaningful classification, but lack of knowledge of the functions of the numerous nucleases impedes its application.

## 2.1. Enzymes hydrolysing both DNA and RNA

### 2.1.1. Enzymes forming nucleoside 5'-phosphates.

Uzawa (1932) first demonstrated the presence of a



Figure 2.

The mode of action of nucleases.

An exonuclease producing 5'-mononucleotides, e.g. snake venom phosphodiesterase, will hydrolyse the bonds 7, 5, 3 and 1 successively.

An exonuclease producing 3'-mononucleotides, e.g. spleen phosphodiesterase, will hydrolyse the bonds 2, 4, 6 and 8 successively.

An endonuclease showing a preference for pyrimidine bases on the 3' side, e.g. pancreatic RNase, will hydrolyse the bonds 4 and 6.

(From Davidson, J. N., "The Biochemistry of the Nucleic Acids", 4th Ed., Methuen & Co. Ltd., London, 1960).



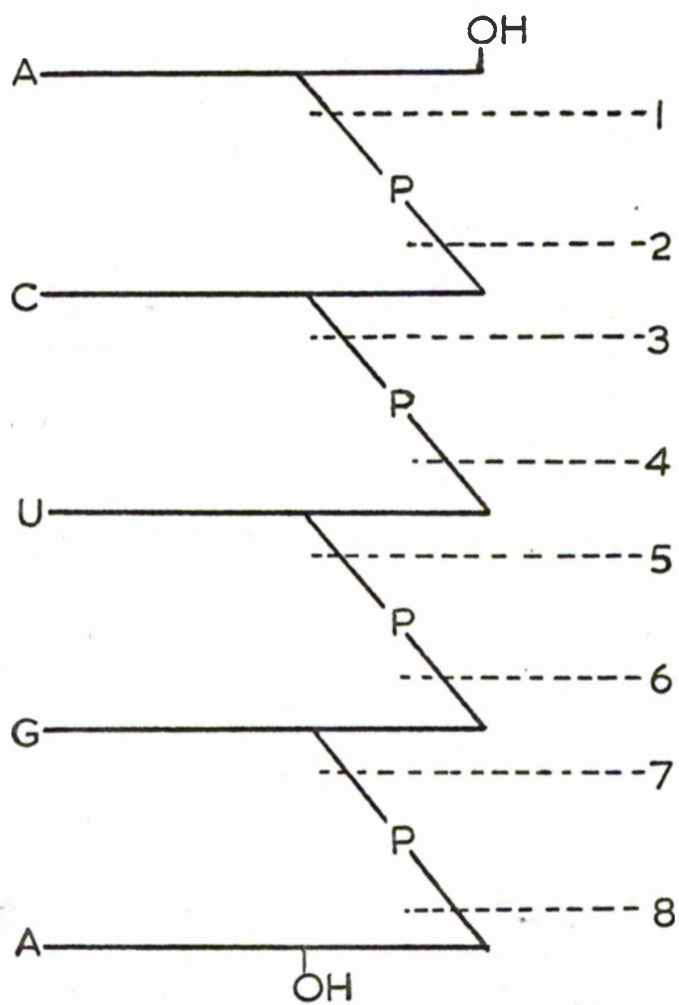


Figure 2.

phosphodiesterase in a snake venom, and it was Gulland & Jackson (1938) who first showed that the enzyme hydrolysed RNA producing ribonucleoside 5'-phosphates. These results were later confirmed by Cohn & Volkin (1953).

Several workers have described methods for the partial purification of snake venom phosphodiesterase (Hurst & Butler 1951; Privat de Garilhe and Laskowski, 1955a; Koerner & Sinsheimer, 1957; Boman and Kaletta, 1957; Bjork & Boman; 1959; Razzell and Khorana, 1959; Laskowski, 1959; Felix, Potter & Laskowski, 1960; Williams, Sung & Laskowski, 1961; Sulkowski & Laskowski, 1962; Bjork, 1963). It is only in the most recent methods that complete removal of phosphomonoesterase (E.C. 3.1.3.) has been achieved. This is important if snake venom phosphodiesterase is to be used in the study of the structure and sequence of oligonucleotide

The pH optimum is between 8.9 - 9.8. The enzyme requires  $Mg^{2+}$  or  $Ca^{2+}$  ions, and is inhibited by high NaCl and substrate concentration (Razzell & Khorana 1959a; Williams, Sung, & Laskowski, 1961).

Boman & Kaletta (1956, 1957) first showed that the enzyme could hydrolyse thymus DNA, and their observations were confirmed by Williams et al. (1961) who demonstrated that the purified phosphodiesterase quantitatively degrades intact



thymus DNA to mononucleotides. Razzell & Khorana (1959a, b) investigated the substrate requirements of the enzyme using synthetic thymidine derivatives. They showed that the enzyme hydrolyses deoxyribose derivatives more rapidly than ribose derivatives; thus p-nitrophenyl-pT is hydrolysed at times the rate of p-nitrophenyl-pU. The presence of phosph on the 3'-hydroxyl of an oligonucleotide completely inhibits the activity, whereas the 3'-acetyl derivative is hydrolysed slowly. Esterification of the 5'-hydroxyl group with phosphate stimulates the activity by a factor of 20. The enzyme hydrolyses polydeoxyribonucleotides and polyribonucleotides terminated by 3'-phosphate groups very slowly. Macrocyclic oligonucleotides, in which the linear oligonucleotides are cyclised by esterifying the 3'-hydroxyl group with the 5'-phosphate group at the other end of the chain, are similarly hydrolysed very slowly (Volkin & Cohn, 1953; Turner & Khorana, 1959; Razzell & Khorana, 1959b). These observations suggest that the snake venom phosphodiesterase contains some endonuclease activity, as does the splitting of oligonucleotides with a terminal 3'-phosphate group when a nucleoside 3',5'-diphosphate is released last rather than first (Felix et al., 1960). Kinetic studies on polyribonucleotides (Singer, Hilme & Heppel, 1958) and on synthetic oligodeoxyribonucleotides (Razzell & Khorana, 1959) bearing terminal 3'-hydroxyl groups have indicated that



hydrolysis begins at the 3'-hydroxyl end and proceeds stepwise along the chain releasing 5'-mononucleotides. It is thus predominantly an exonuclease.

A similar nuclease activity has been demonstrated in a number of tissues of the rat (Razzell, 1961). This activity is localised in the microsome fraction, and has been purified from hog kidney approximately 300 fold. It has essentially the same properties and specificities as snake venom phosphodiesterase. Anderson & Heppel (1960) have partially purified and characterised an exonuclease from a leukaemic cell line. This enzyme is essentially similar to snake venom phosphodiesterase except it requires the presence of two nucleosides on both sides of the phosphate bridge. Thus it does not hydrolyse synthetic substrates such as p-nitrophenyl-pT.

An endonuclease hydrolysing both DNA and RNA has been partially purified by Stevens & Hilme (1960) from Azotobacter agilis. This enzyme has a pH optimum of 7.7, and requires  $Mg^{2+}$  ions.  $Mn^{2+}$  and  $Co^{2+}$  ions also stimulate the activity. The main products of digestion are dinucleotides, trinucleotides and tetranucleotides with 5'-phosphate end groups. Another type of enzyme falling in this group has been isolated from mung bean sprouts (Sung & Laskowski, 1962). Though this enzyme gives similar products, it does have a partial exonucleolytic activity and differs in its pH



optimum at 5.0 and in having no  $Mg^{2+}$  requirement. It does show some specificity towards the bases and hydrolyses preferentially the phosphodiester linkage in the sequence pApX.

#### 2.1.2. Enzymes forming nucleoside 3'-phosphates.

Calf spleen contains several nucleases, one of which Heppel & Hilme (1953) identified as an exonuclease. The exonuclease was first purified by Hilme (1960) and Razzell & Khorana (1961) have since improved the method. The pH optimum of the enzyme is about 6.5. It shows no requirement for and is indeed inhibited by divalent cations.

The enzyme hydrolyses both RNA and DNA to give nucleoside 3'-phosphates (Heppel, Whitfield & Markham, 1953; Heppel, Ortiz & Ochoa, 1957). Razzell & Khorana (1961) using synthetic oligodeoxyribonucleotides showed that the enzyme acted in a complementary manner to snake venom phosphodiesterase by hydrolysing the chain from the 5'-hydroxyl end giving 3'-mononucleotides in a stepwise fashion. The 5'-hydroxyl position must be unsubstituted and the presence of a 5'-phosphate group inhibits the reaction completely. However substitution at the 3'-hydroxyl does not affect the rate of hydrolysis. The enzyme is also capable of transfer reactions such as the formation of dTpTpT and dTpTpTpT by incubation of a high concentration of dTpT with



the enzyme.

Razzell (1961) using Tp-nitrophenyl as a substrate for phosphodiesterase activity showed that enzymes of this type are widely distributed in animal tissues. In contrast to the activity towards the 5' isomer, p-nitrophenyl-pT, the activity is found predominantly in the supernatant and mitochondrial fractions of a cell free extract. A similar enzyme has been partially purified from Lactobacillus acidophilus

Micrococcal nuclease (E.C.3.1.4.7) is the trivial name for an enzyme found in Micrococcus pyogenes (Cunningham, Catlin & Privat de Garilhe, 1956) which hydrolyses DNA and RNA to yield oligonucleotides terminated with 3'-phosphate groups. The partial purification of the enzyme has been described by Pochon & Privat de Garilhe (1960) and by Alexander, Heppel & Hurwitz (1961). The enzyme has a pH optimum at 8.6. It is completely dependent on  $\text{Ca}^{2+}$  ions and is heat stable. The products of digestion of DNA and RNA contain 30% and 60% mononucleotides respectively suggesting the presence of exonuclease activity. Pochon & Privat de Garilhe (1960) have proposed that there are two separate enzymes in the purified preparation. But Alexander et al. (1961) and Williams et al. (1961) have demonstrated a predominantly endonucleolytic activity and Sulkowski &



Laskowski (1962) showed that dinucleotides were completely resistant to the preparation. The enzyme shows a preference for d-XpTp- and d-XpAp- bonds (Rushizky, Knight, Roberts & Dekker, 1960; Pochon & Privat de Garilhe, 1960) and a preference for denatured DNA over native DNA (Dirksen & Dekker, 1960). This preference together with the heat stability of the enzyme have been utilised by von Hippel & Felsenfeld (1964) to study DNA conformation. They showed that at 60° denatured DNA was attacked essentially at random while the initial attack on native DNA was in regions rich in dAdo and dThd residues. This initial attack is presumably the result of a) the preference for d-XpTp- and d-XpAp- b) the loosening of the helix in regions rich in dAdo and dThd. They suggested that considerable insight into some aspects of local conformation of nucleic acids might be gained with the use of enzymes such as micrococcal nuclease.

## 2.2. Enzymes hydrolysing DNA

The grouping of DNases into those forming 5'-monoesters and those forming 3'-monoesters coincides with a division of DNases before these specificities were known, that is division into DNase type I (E.C.3.1.4.5) and DNase type II (E.C.3.1.4.6) as exemplified by pancreatic DNase I (Kunitz, 1948) and thymic DNase II (Maver & Greco, 1949) respectively. These two types



were introduced to compare and contrast the types with respect to pH optima, activating and inhibitory agents. At the time little was known of their specificities. Later it was shown that in general the DNase I type of enzyme produce 5'-monoesters, and DNase II type of enzyme produce 3'-monoesters, though there are a few exceptions. This terminology is still useful as long as a DNase type I is recognised as meaning similarity to rather than chemical identity to pancreatic DNase I.

2.2.1. Enzymes forming deoxyribonucleoside 5'-phosphates.

The classical type is the crystalline enzyme from bovine pancreas (Kunitz, 1948, 1950). The enzyme has a broad pH optimum about 7.0. It has a requirement for a divalent cation,  $Mg^{2+}$  or  $Co^{2+}$  (Laskowski & Seidel, 1945; McCarty, 1946; Greenstein, Carter & Chalkley, 1947), and  $Ca^{2+}$  ions have a powerful synergistic effect in the presence of  $Mg^{2+}$  ions (Wiberg, 1958), though this effect has been questioned by Shack & Bynum (1964). The secondary structure of the substrate affects the rate of hydrolysis, for the enzyme hydrolyses native DNA more rapidly than denatured DNA as measured by methyl green binding (Kurnick, 1954) or release of protons Dirksen & Dekker, 1958).

Exhaustive digestion of DNA gives a mixture of mononucleotides and oligonucleotides, whose chain lengths



vary upto 8 units. These products were identified as being terminated by 5'-phosphate groups, by their susceptibility to 5'-nucleotidase and venom phosphodiesterase (Sinsheimer & Koerner, 1951, 1952; Potter, Brown & Laskowski, 1952; Sinsheimer, 1954; Privat de Garilhe & Laskowski, 1955). Identification of the dinucleotides showed that the linkages d-pPupPy - were less evident than random hydrolysis would suggest (Sinsheimer, 1955; Privat de Garilhe, Cunningham, Laurila, & Laskowski, 1957), and digestion of trinucleotides of known sequence supported the hypothesis that the d-pPupPy-linkage is preferentially attacked (Potter, Laurila & Laskowski, 1958; Potter & Laskowski, 1959). However work with synthetic oligonucleotides of the type dTpTpTpTpT and dTpTpTpTpTp has shown that the proximity of the monoesterified phosphate group and the length of the chain are responsible for the labilisation of the linkage rather than the length of the chain alone (Khorana & Gilman, 1959; Khorana, 1959). The endonucleolytic attack is indicated by the result that the sum of the four mononucleotides obtained after exhaustive digestion amounts to only 1% of the total nucleotides present (Sinsheimer, 1954).

An enzyme resembling DNase I with respect to the pH optimum and  $Mg^{2+}$  requirement has been detected in a number of tissues of the mouse (Shack, 1957). This enzyme has been



partially purified from rat liver (Burdon, Smellie & Davidson, 1964) and from lamb brain (Healey, Stollar, Simon & Levine, 1963). However these two activities differ from pancreatic DNase I in the same respect, they hydrolyse denatured DNA more rapidly than native DNA.

Streptococci of serological groups A and C produce substantial amounts of extracellular DNase. Wannamaker (1958, 1962) and Winter & Bernheimer (1964) have demonstrated the separation of four distinct enzymes, which they have termed A, B, C and D. DNase A is more commonly known as streptodornase, which is available in a purified form commercially from the Lederle Division of the American Cyanamide company. A further purification of this material has been obtained (Georgatos, Unterholzner & Laskowski, 1962). The enzyme resembles pancreatic DNase I in pH optimum,  $Mg^{2+}$  requirement (Tillet, Sherry & Christensen, 1948; McCarty, 1948) and the synergistic effect of  $Ca^{2+}$  ions in the presence of  $Mg^{2+}$  ions (Stone & Burton, 1961). Native DNA is hydrolysed at approximately twice the rate of denatured DNA and the products are oligonucleotides terminated by 5'-phosphate. Potter & Laskowski (1959) tentatively concluded that the enzyme preferentially attacks d-pPpPu-linkages. Examination of the bases terminating the oligonucleotides showed that dGua predominated at the 5' end, whereas there



was random distribution of the bases at the 3' end (Georgatos et al., 1962). Winter & Bernheimer (1964) separated DNase B, C and D by continuous paper electrophoresis and sucrose gradient electrophoresis. DNase B resembles DNase A with respect to the pH optimum, the effect of  $Mg^{2+}$  and  $Ca^{2+}$  ions and the specificity. DNase C and D have slightly more acid pH optima. The specificity of DNase D complements that of DNases A and B in that it gives a predominance of dAdo at the terminal 5'-hydroxyl position. DNase C shows no special specificity.

Neurospora crassa contains two enzymes termed I and II (Linn & Lehman, 1964). DNase II has a pH optimum at 7.5, and requires  $Mg^{2+}$  ions. However it hydrolyses denatured DNA more rapidly than native DNA, producing on exhaustive digestion a mixture of mononucleotides, dinucleotides, trinucleotides and tetranucleotides. The mononucleotides are sensitive to 5'-nucleotidase, and are presumably the 5' isomers. DNase I attacks native DNA at a pH optimum of 5.3 and requires  $Mn^{2+}$  ions. No further information is available to enable further classification.

There are at least four distinct DNA specific enzymes in E. coli. They all give on digestion either deoxyribonucleoside 5'-phosphates or oligonucleotides terminating in 5'-phosphate groups. Three of these enzymes



are exonucleases that Lehman (1964) has termed I, II and III. The other enzyme is an endonuclease that has been partially purified by Lehman, Reussos & Pratt (1962). This enzyme has a pH optimum between 7.5 - 8.5, and a requirement for  $Mg^{2+}$  ion. It hydrolyses native DNA seven times more rapidly than denatured DNA giving oligonucleotides terminating in 5'-phosphate groups whose average chain length is seven. The activity is inhibited by RNA, most effectively by E. coli SRNA to which it is found bound in crude extracts. This property distinguishes it from most other types of DNases.

Exonuclease I (Lehman, 1960) is specific for denatured DNA which it digests from the 3'-hydroxyl producing 5'-mononucleotides until the terminal dinucleotide is reached. This terminal dinucleotide is resistant to further hydrolysis. The enzyme is thus a useful tool for the identification of the terminal base of a chain (Lehman & Nussbaum, 1964). It has a pH optimum of 9.2 - 9.8 and shows an absolute requirement for  $Mg^{2+}$  ions.

Exonuclease II (Lehman & Richardson, 1964) differs from exonuclease I in two respects (i) it is specific for native DNA (ii) it hydrolyses dinucleotides. But its pH optimum and  $Mg^{2+}$  requirement are the same. An unusual feature of this enzyme is its close physical relationship to the DNA polymerase of E. coli (E.C.2.7.7.7.) from which it has not



yet been dissociated. Lehman & Richardson (1964) however point to a number of features of catalytic specificity that suggest the two activities do not belong to the same protein. The finding of Okazaki & Kornberg (1964) that in Bacillus subtilis the DNA polymerase can be obtained practically free of exonuclease II supports the contention that the exonuclease is not obligatory for the functioning of DNA polymerase.

Exonuclease III (Richardson, Lehman & Kornber, 1964) appears to be inseparable from a highly specific phosphatase. The phosphatase activity requires a 3'-phosphomonoester on a polydeoxyribonucleotide chain; it does not attack short oligonucleotides phosphorylated on the 3'-hydroxyl group. This activity thus allows the hydrolysis of chains terminated by a 3'-phosphate group which blocks or inhibits the activity of the two other exonucleases. The exonuclease III is specific for native DNA, but differs from exonuclease II in that digestion proceeds only to about 40% of completion. This incomplete digestion may be explained by the insusceptibility of single stranded DNA. Thus hydrolysis will proceed from the 3'-hydroxyl of both ends leaving a single stranded DNA which is then resistant to further hydrolysis. The enzyme has a pH optimum of 7.7; it requires  $Mg^{2+}$  ions and is inhibited by  $Zn^{2+}$  ions, and



p-chloromercuribenzoate.

An additional nucleolytic activity has been described by Jorgensen, Olesen & Koerner (1964). This activity has a pH optimum of 8.5 and requires  $Mg^{2+}$  ions. It neither cleaves nor is inhibited by RNA. It preferentially attacks oligodeoxyribonucleotides giving deoxyribonucleoside 5'-phosphates but it does not cleave highly polymerised single or double stranded DNA. Though its mode of action is not defined with respect to whether it is exonucleolytic or endonucleolytic, it appears to be distinguishable from the endonuclease and the exonucleases I, II and III.

A similar enzyme has been partially purified from extracts of E. coli infected with T2 bacteriophage (Olesen & Koerner, 1964). This enzyme though having equivalent properties is chromatographically distinct, and is far less stable to heat than that described by Jorgensen et al. (1964).

Also from T2 infected E. coli an endonuclease has been partially purified with properties similar to Lehman's endonuclease (Bose & Nossal, 1964). This activity was shown to increase on infection with T2 by a factor of 4 - 6 in 12 minutes. Chromatography of the infected cell extract on DEAE-cellulose revealed a DNase peak with the above mentioned properties that was not present in uninfected cells. This peak was assumed to be phage induced and is presumably



the enzyme identified by Stone & Burton (1962) who presented evidence for the view that it was newly synthesised.

Lysogenic induction of E.coli K12  $\lambda$  brings about the appearance of relatively large amounts of an exonuclease which was not detected before induction (Korn & Weissbach, 1963). Induction of E.coli K12 (434) to give phage 434 resulted in the formation of a physically and enzymatically similar enzyme (Korn & Weissbach, 1964). This activity has a pH optimum of 10.5; it requires  $Mg^{2+}$  ions and is labile to heat. It attacks native DNA approximately 10 times more rapidly than denatured DNA giving 5'-mononucleotides. It does not attack however p-nitrophenyl-pT or RNA. This type of enzyme is not synthesised during infection of E.coli with phage T4 (Weissbach & Korn, 1964) and it may thus represent a class of enzyme related to DNA metabolism that is uniquely required for temperate phage replication. Its precise role however remains to be elucidated.

#### 2.2.2. Enzymes forming deoxyribonucleoside 3'-phosphates.

A DNase which has a pH optimum between 4.5 - 5.5 was observed in spleen by Catcheside & Holmes (1947) and later in thymus by Maver & Graco (1949). Several methods have been described for the partial purification of the enzyme (Webb, 1953; McDonald, 1955; Koszalka, Falkenheim & Altman, 1957; Shimomura & Laskowski, 1957; Koerner & Sinsheimer,



1957; Fredericq & Oth, 1958) but these preparations still contain RNase. Maver, Peterson, Sober & Greco (1959) have described a method giving a preparation free of RNase, and more recently Bernardi (1961) and Bernardi & Griffe (1964) have described procedures for thymus and spleen respectively giving an enzyme consisting of a single protein component as illustrated by chromatographic, electrophoretic and ultracentrifugal techniques.

The pH optimum of the enzyme cannot be clearly defined since it is dependent on the method of estimation, the cations present, and on the ionic strength of the medium (Koerner & Sinsheimer, 1957; Oth, Fredericq & Hacha, 1958). The enzyme requires a monovalent cation at a concentration of 0.2 - 0.3M for maximal activity;  $Mg^{2+}$  ions stimulate the enzyme at concentrations of the order of 0.001M, and the substrate itself usually contains enough  $Mg^{2+}$  to satisfy this requirement. Higher concentrations of  $Mg^{2+}$  ions inhibit (Koerner & Sinsheimer, 1957).

Digestion of DNA produces mononucleotides, dinucleotides and higher oligonucleotides, all terminating in 3'-phosphate groups (Privat de Garilhe & Laskowski, 1955). It has not yet been determined unequivocally whether any linkage is preferentially attacked. Koerner & Sinsheimer (1957) concluded that the enzyme showed no preferences, but Laurila



& Laskowski (1957) suggested that d-pPyrpPu- is more sensitive than others.

Using the pure enzyme from spleen, Bernardi & Sadron (1964) have studied the kinetics of the degradation of native DNA. Pancreatic DNase I attacks native DNA according to double hit kinetics only, i.e. Scission of a single chain and thus for breakage of the chain two hits are necessary at approximately the same position. But Bernardi & Sadron (1964) have concluded that DNase II degrades native DNA initially by single hit kinetics, i.e. scission of both chains at the same level and latterly by both single and double hit kinetics.

DNase II activity has been demonstrated in a number of other tissues (Shack, 1957) and several investigators have shown a correlation between growth and this activity; for example Brody & Balis (1959) in regenerating rat liver, Solomon (1964) in developing mouse embryo, and Coleman (1963) during amphibian metamorphosis. In the last case, the enzyme presumably plays an important part in the resorption of the tadpole's tail.

Other DNases have been reported, but with insufficient data to classify them; Carlson & Frick (1964) from garlic seedlings; Linn & Lehman (1964) from N. crassa.



## 2.3. Enzymes hydrolysing RNA

The RNases are widespread and have been identified in animal, bacterial and plant cells. Most of these have certain common properties:- 1) heat stability 2) hydrolysis of RNA by a two step process involving firstly the transesterification of the phosphodiester bond in RNA to give 2', 3'- cyclic phosphate terminated oligonucleotides, and secondly the hydrolysis of the 2', 3'-cyclic phosphate (Brown & Todd, 1955) 3) the pH optima fall into two groups, one with optima between 4 - 6.0 which are mainly obtained from plants and one with optima between 7.0 - 8.0 which are mainly obtained from animals.

### 2.3.1. Enzymes forming ribonucleoside 3'-phosphates.

Much that is known about this type of enzyme comes from the study of pancreatic RNase. Bovine pancreatic RNase was first crystallised by Kunitz (1940) but was subsequently isolated in a highly purified form by Hirs, Moore & Stein (1953) by column chromatography. The amino acid sequence of the enzyme was determined by Hirs, Moore & Stein (1960).

The pH optimum seems to depend on 1) source of RNA 2) ionic strength. Using rat liver RNA as a substrate Kaver & Greco (1956) obtained a value of 6.5, whereas Kunitz (1940) reported a pH optimum of about 7.6 with yeast RNA as substrate. The optimal ionic strength was reported to be 0.1M (Dickman, Aroskar & Kropf, 1956) but Dickman & Ring



(1958) showed that a higher value was optimal for the synthetic substrates, cytidine and uridine 2',3'-cyclic phosphates. Divalent ions generally inhibit the enzyme (Houck, 1957). The specific structural requirement for the action of the enzyme is a pyrimidine ribonucleoside 3'-phosphoryl group (Brown & Todd, 1955). The enzyme is essentially unspecific with respect to the nature of the group esterified to the 3'-phosphoryl, so that the enzyme cannot be classified as exclusively endonuclease or exonuclease.

The purity, heat stability of the enzyme and the availability of defined substrates have made it possible to investigate the kinetics of the enzyme, and from such studies a more complete understanding of the mechanism of action has been achieved (review: Witzel, 1964).

The spleen and liver of the calf and rat have been extensively studied with respect to their RNase content. An enzyme was purified from calf spleen (Kaplan & Heppel, 1956). This enzyme was shown to resemble pancreatic RNase except that its pH optimum was between 6.0 - 6.5, but later several peaks of RNase activity were obtained by chromatography on DEAE-cellulose (Maver et al., 1959). Maver & Greco (1962) have since separated two activities from each other and from



contaminating phosphomonoesterase and phosphodiesterase activities. One has a pH optimum of 5.7; it is slightly inhibited by  $Mg^{2+}$  ions which also lower the pH optimum to 5.0, and it is heat labile. Digestion of RNA did not produce a "core", and the products included oligonucleotides terminated by 2',3'-cyclic phosphate and 3'-phosphate groups. These results are in contrast to the properties of pancreatic RNase and splenic RNase isolated by Kaplan & Heppel (1956). The other activity has a pH optimum of 7.0; it is stimulated by  $Mg^{2+}$  ions and is relatively heat stable. On digestion of RNA this enzyme leaves an undigested "core" together with oligonucleotides. It does not appear to have an absolute specificity for pyrimidine bases.

The existence of two RNase activities in the liver of the rat was first demonstrated by de Lamirande, Allard, da Costa & Cantero (1954). The acid RNase was partially purified by Roth (1957) from the rat, and subsequently by Maver & Greco (1962) from the calf. The enzyme has a pH optimum about 6.0; it is activated slightly by  $Mg^{2+}$  ions, and is heat labile (Roth, 1957; Zytko, de Lamirande, Allard & Cantero, 1958). The enzyme shows no specificity towards the bases, leaving no 'core' and producing both purine and pyrimidine 2',3'-cyclic phosphates and 3'-phosphates (Reid & Niles, 1959; Maver & Greco, 1962). The enzyme thus



resembles the activity obtained from calf spleen by Waver & Greco (1962). The alkaline RNase has been extensively purified (Roth, 1957). It has a pH optimum of 7.8, and a high heat stability. Its specificity would appear to be similar to pancreatic RNase, since it produces an undigested core on digestion of RNA, and does not attack the pancreatic RNase 'core'. Among the products there are only pyrimidine 2',3'-cyclic phosphates, and the enzyme does not hydrolyse these further to 3'-phosphates (Zytko et al., 1958; Reid & Nodes, 1959). However Beard & Razzell (1964) have suggested that the preparation used contained an inhibitor of the hydrolysis of pyrimidine 2',3'-cyclic phosphates.

Many plant tissues contain RNase activity, but in only a few cases has the enzyme involved been purified and characterised. From soybean seedlings a RNase was partially purified by Merola & Davis (1962). Like pancreatic RNase it requires a high salt concentration for optimal activity, and it is heat stable. However it shows no specificity similar to pancreatic RNase. The products of digestion contain purine and pyrimidine 2',3'-cyclic phosphates and purine 3'-phosphates. An enzyme very similar to that from soybean seedlings is one described by Wilson (1963) purified from corn seedlings. However this enzyme is unable to hydrolyse any of the four cyclic phosphates. Ryegrass seedlings



provide another enzyme capable of hydrolysing RNA to 2',3'-cyclic phosphates, followed by slow hydrolysis to the 3'-phosphates (Shuster, Khorana & Heppel, 1959). This preparation contains an inhibitor of the hydrolysis of pyrimidine 2',3'-cyclic phosphates. This inhibitor however can be removed, and it suggests the possibility of its presence in soybean seedlings. Other similar enzymes have been partially purified from pea leaf (Holden & Pirie, 1955) Markham & Strominger, 1956) and tobacco leaf (Frish-Niggermeyer & Reddi, 1957).

E.coli contains two distinct RNases. Both activities have been characterised. One produces oligonucleotides terminated in 5'-phosphate group, and will be discussed in the next section. The other enzyme has been partially purified by Spahr & Hollingsworth, (1961). This enzyme is located in the ribosomes in a latent form; it can be released from the ribosomes by destruction of their structure by removal of  $Mg^{2+}$  ions or addition of urea (Elson, 1959). The enzyme appears to be confined to the 30S particles, and occurs at approximately 0.1 molecule per particle. It would thus appear to be unnecessary for the functioning of the ribosome (Spahr & Hollingsworth, 1961). The enzyme has a pH optimum of 8.1. It requires a high salt concentration for optimal activity, but it is inhibited by  $Mg^{2+}$  ions.



Digestion of RNA shows that it cleaves all bonds in RNA giving 2',3'-cyclic phosphates which are slowly hydrolysed to 3'-phosphates. RNase activity has also been detected in the microsomes of rat liver (Tashiro, 1958) and in nuclear ribosomes of calf thymus (Wang & Wang, 1962).

RNase T<sub>1</sub> (E.C.3.1.4.8) is an enzyme basically similar to pancreatic RNase. It can be purified from takadiastase, a commercial powder prepared from Aspergillus oryzae which contains several RNases. The complete purification and the physical properties have been described (Egami, Takahashi & Uchida, 1964). The primary structure has been partially determined, and there would appear to be little similarity between RNase T<sub>1</sub> and pancreatic RNase. RNase T<sub>1</sub> has a pH optimum of 7.5, and it is slightly inhibited by  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  ions. EDTA has a stimulatory effect. The enzyme is as stable a protein as pancreatic RNase. However RNase T<sub>1</sub> has a specificity distinct from pancreatic RNase, for it splits only the internucleotide bonds between 3'-GMP and the 5'-hydroxyl of the adjacent nucleotides with the intermediary formation of guanosine 2',3'-cyclic phosphate (Sato-Asano, 1959). Similar enzymes have been identified in B. subtilis (Rushizky, Greco, Hartley & Sober, 1963), Streptomyces albrogriseolus (Yoneda, 1964), Streptomyces erythreus (Tanaka & Cantoni, 1963) and Ustilago sphaerogena



(Glitz & Dekker, 1964).

A second RNase, RNase T<sub>2</sub>, has been studied from takadiastase (Kigami et al., 1964). This enzyme differs from RNase T<sub>1</sub>, in several respects. Its amino acid composition has been determined and it shows that RNase T<sub>2</sub> is a neutral protein compared with the acidic nature of RNase T<sub>1</sub>. The molecular weight of RNase T<sub>2</sub> is approximately three times that of RNase T<sub>1</sub>, whose molecular weight is comparable with pancreatic RNase. It has a pH optimum of 4.5, and is inhibited by  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  ions. RNase T<sub>2</sub> however is somewhat less stable than RNase T<sub>1</sub>, though it does not lose any activity when heated to 80° for 5 minutes at pH 6.0. The enzyme attacks all the internucleotide bonds in RNA but with a preference for Ado (Ruskizky & Sober, 1963).

#### 2.3.2. Enzymes forming ribonucleoside 5'-phosphates.

There are few activities specific for RNA producing nucleotides terminated by 5'-phosphate groups. These known come mainly from bacterial sources, E.coli, L.casei and S. albobrassicolus. Spahr (1964) has partially purified from E.coli a RNase found in the supernatant and ribosomal fraction of cell extracts. This enzyme has a pH optimum of 7.0 - 8.0. It requires both monovalent and divalent cations for optimal



activity, and is unstable. It appears to attack RNA exonucleolytically and endonucleolytically. Spahr (1964) has suggested that the enzyme is that which Wade & Lovett (1961), Sekiguchi & Cohen (1963) and Singer & Tolbert (1964) have identified in E. coli extracts. A comparable enzyme has been partially purified from L. casei (Keir, Mathog & Carter, 1964). It would appear to differ in that it requires specifically  $K^+$  ions for optimal activity.

An endonuclease has been partially purified from S. albobrassicolus (Yoneda, 1964). This enzyme has a pH optimum of 6.5 - 9.0; it requires  $Mg^{2+}$  or  $Mn^{2+}$  ions. The specificity of the enzyme has been studied, but no apparent specificity towards the bases has been demonstrated. The partial purification of an endonuclease from the nucleic fraction of guinea pig liver has been reported (Heppel, Ortiz & Ochoa, 1956; Razzell, 1963). This enzyme hydrolyses poly A and poly U to oligonucleotides of two to six units in length, terminated in 5'-phosphates.

### 3. Cellular Inhibitors of Nucleases.

The existence of inhibitors of nucleases would appear essential as active intracellular nucleases must act mainly to the detriment of the cell. The presence of an inhibitor to DNase and RNase has been demonstrated in many different



tissues and different animals.

### 3.1. RNase Inhibitor.

Such an inhibitor has been partially purified and characterised from rat liver (Roth, 1958; Shortman, 1962). The inhibitor is inactivated by low concentrations of papain, periodate, sulphhydryl reagents, protamine and high salt concentrations. The available evidence suggests the inhibitor is protein, but also that carbohydrate is an important component. The inhibitor may be classed as polyanionic as it has a high affinity for DEAE-cellulose. This inhibitor acts only on alkaline RNase, having little effect on acid RNase. A RNase inhibitor has also been indicated in rat adipose (Kichel, Figueroa & Goldenberg, 1961) rat uterine tissue (Roth, 1962) mouse and bovine pancreas (Dickman, Merrill & Trupin, 1960) and in the liver of mouse, hamster and guinea pig (Roth, 1962).

### 3.2. DNase Inhibitors.

Two different types have been described. One type is polyanionic, the other apparently protein. The polyanionic inhibitor acts solely on DNase type II of animal tissues and is presumably due to the basic character of DNase type II, for the inhibition is decreased by increasing salt concentration (Tunis & Hegelson, 1963). RNA can act



similarly as an inhibitor presumably in a similar manner (Bernardi, 1964; Jacquemin-Sablon, Laval, Le Talaer, Le Pecq & Paoletti, 1964). Such inhibition is unlikely to be a physiological effect as there is considerable evidence that DNase type II is normally confined to lysosomes (Beaufray, Bendall, Bandlin & Duve, 1959). However Lehman, Roussos & Pratt (1962) have described the properties of a RNA inhibitable endonuclease. The endonuclease is present in the supernatant of cell extracts, and the most effective inhibitor is E.coli sRNA.

Inhibitors of DNase type I appear to be widespread (Cooper, Trautman & Laskowski, 1950). The properties of the inhibitor from rat liver have been described (Loiselle & Carrier, 1963; Zalite & Roth, 1964). This inhibitor acts on pancreatic DNase I and alkaline DNase in rat liver mitochondria. Evidence is presented demonstrating the binding of the inhibitor to the DNA as its mode of inhibition. However an inhibitor partially purified from calf spleen (Lindberg, 1964) appears to inhibit pancreatic DNase I by binding to the enzyme. This inhibitor also has the general properties of protein.

The present work was undertaken to obtain a more complete



understanding of the properties and specificity of a nuclease from rat liver which preferentially attacks denatured DNA and initially described by Burdon et al. (1964). It was anticipated that such information would have a two fold value since 1) the nuclease may have such a specificity as to be of some value in studying nucleic acid structure and sequence, 2) the knowledge may provide some clue to the problem of the possible physiological functions of a nuclease.



## CHAPTER II

### The Partial Purification and Properties of the Liver Nuclease.



1. Introduction.

The further purification of the nuclease characterised by Burdon et al. (1964) was considered necessary if its properties and specificity were to be examined in detail. The partially purified nuclease still contained RNase, phosphodiesterase I and II and phosphomonoesterase. The procedure of Burdon et al. (1964) consisted of the preparation of an aqueous extract of rat liver which was fractionated by acid and ammonium sulphate precipitation followed by acetone fractionation. Other methods that were investigated involved the use of calcium phosphate gel, alumina c<sub>8</sub> gel, CM-Sephadex, CM-cellulose, DEAE-Sephadex and DEAE-cellulose, and 3-4 fold increases in the specific activity of the nuclease were obtained. No further purification was achieved by gel filtration on Sephadex G-200 for the enzyme was eluted along with the bulk of the protein in the void volume indicating either that the enzyme had a molecular weight in excess of 200,000 or that aggregate molecules involving the enzyme were present.

It was therefore considered that the contaminating activities, RNase, phosphodiesterase I and II and phosphomonoesterase, might be partially removed by first carrying out the fractionation of the subcellular components, since the work of Beaufray et al. (1959) had shown that



alkaline DNase was located in the mitochondria, and the activity towards denatured DNA was similarly located (Burdon, 1963). These results showed that the supernatant fraction of rat liver contained very little alkaline DNase, so it was assumed that the procedure adopted for the preparation of the aqueous extract resulted in the release of alkaline DNase from the mitochondria. Therefore the mitochondria were utilised as a source of the nuclease in place of the aqueous extract.

The subsequent work described in this chapter shows that the physical properties of the nuclease obtained from rat liver mitochondria differ significantly from those of the enzyme as it occurs in soluble extracts of whole rat liver. Thus the enzyme from mitochondria does not respond in the same way to the purification procedures applied to the soluble extracts. For example, the mitochondrial enzyme could not be precipitated by lowering the pH and this procedure was eliminated. The remaining two steps, ammonium sulphate and acetone fractionations, required some adjustments in order to achieve significant increases in specific activity. These preliminary findings however illustrate two important points concerning the purification of proteins. Firstly, the source of the protein is worthy of careful consideration, and secondly, the fractionation procedures depend to a large



extent on the particular proteins present, so that if the starting material is changed the fractionation procedures may not function as efficiently as before.



## 2. Experimental

### 2.1. Materials

DNA from both Landschutz ascites carcinoma cells and calf thymus glands was used through the course of this investigation. The two types of DNA gave identical results in assays for nucleases. Extracts of Landschutz ascites carcinoma cells were prepared by the method of Keir, Binnie & Smellie (1962) and, from the sediment obtained by the centrifugation of such extracts at  $105,000 \times g$  for 1 hr., DNA was prepared by the method of Kay, Simmons & Dounce (1952). DNA was also prepared from calf thymus glands by this method. The DNA was usually dissolved in water to give a final concentration of 2mg./ml.

Calf thymus DNA purchased from the Sigma Chemical Company, St. Louis, Mo., U.S.A., and salmon sperm DNA from the California Corporation for Biochemical Research, Los Angeles, California, U.S.A., were used in a few specified experiments. Heat denatured DNA was prepared by heating a solution of DNA in water at a concentration of 2mg./ml. in a boiling water bath for 10 min., and then by cooling rapidly by placing in an ice bath.

Highly polymerised yeast RNA was purchased from the British Drug Houses, Poole, Dorset, and was dissolved in water to give a solution containing 2mg./ml. Yeast sRNA



was kindly supplied by Dr. D. Bell. It was prepared by the method of Bell, Tomlinson & Tener (1964). Ultracentrifugation and melting curve behaviour indicated that the sRNA was most probably double stranded.

p-Nitrophenyl phosphate (disodium salt) was purchased from the British Drug Houses, and p-nitrophenyl-pT (monosodium salt) from the California Corporation for Biochemical Research. Tp-nitrophenyl (mon ammonium salt) was synthesised by the method of Turner & Khorana (1959).

Triton-X-100 was purchased from Rohm & Hass, Philadelphia, P.A., U.S.A., 2-mercaptoethanol from L.Light and Company, Ltd., Colnbrook, Bucks, iodoacetamide from the British Drug Houses, sodium p-hydroxymercuribenzoate from the Sigma Chemical Company, DEAE-cellulose (DE 11 fibrous powder with a nominal capacity of 1.0 mequiv./g.) and OM-cellulose (OM70, powder with a nominal capacity of 0.7 mequiv./g.) from Reeve Angel & Company, Ltd., London, the unchanged dextran gels, Sephadex G-75 and G-200, the charged dextran gels, DEAE-Sephadex (A50, medium) and OM-Sephadex (C50, medium) and Blue Dextran 2000 from Pharmacia, Uppsala, Sweden, cytochrome C (horse heart) from Seravac Laboratories, Colnbrook, London, bovine  $\gamma$ -globulin and serum albumin from the Armour Pharmaceutical Company, Eastbourne, Sussex, sintered polythene from Fisons Scientific Apparatus Ltd., Leicestershire. Other



reagents were analar grade, except tris and imidazole which were laboratory reagents.

## 2.2. Buffer solutions

Tris buffers were prepared by titration of 1M tris solution to the required pH with concentrated HCl, followed by dilution to the required molarity. Imidazole buffers were prepared similarly by titration of 0.5M imidazole solution with concentrated HCl. Acetate buffers were prepared by titration of 0.5M acetic acid with 0.5M sodium acetate to the required pH.

## 2.3. Methods of estimation

### 2.3.1. Protein.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as a standard.

### 2.3.2. Phosphorus

Total phosphate and inorganic phosphate were estimated by a modification of the method of Allen (1940). For total phosphate, the sample was digested with 0.24 ml. 10N  $\text{H}_2\text{SO}_4$ . Digestion was continued until the material was fuming. Coloured material was removed by the addition of a drop of hydrogen peroxide followed by heating again until the digest was fuming. This procedure was repeated until a clear liquid was obtained. Any remaining hydrogen peroxide,



which interferes with the method of estimation, was removed by adding a small volume of water. The solution was then heated until it reached the stage of fuming. It was then allowed to cool. It was assumed that 0.24 ml. 10N  $\text{H}_2\text{SO}_4$  gave 0.07 ml. of the final digest. Water was added to give a volume of 4.4 ml. followed by 0.2 ml. 8.3% (w/v) ammonium molybdate and 0.4 ml. 1% (w/v) amidol in 20% (w/v) sodium metabisulphite. The solution was mixed and allowed to stand for at least 10 min. but not longer than 30 min. The extinction at 638.6 m $\mu$  was measured.

Inorganic phosphate was estimated as above except the digestion was eliminated. Thus to the sample water was added to bring the volume to 4.16 ml. 0.24 ml. 10N  $\text{H}_2\text{SO}_4$  was added followed by 0.2 ml. 8.3% (w/v) ammonium molybdate and 0.4 ml. 1% (w/v) amidol in 20% (w/v) sodium metabisulphite. The extinction at 638.6 m $\mu$  was measured after the solution had been standing for 10 min.

### 2.3.3. Ammonium salts.

Ammonium salts were estimated using a commercial Nessler's reagent (British Drug House). The solution to be estimated was made up to a total volume of 7 ml. with water and 1 ml. Nessler's reagent was added. After mixing the solution was allowed to stand for a few minutes. The extinction at 490 m $\mu$  was measured. This procedure was



satisfactory for samples containing 0.005 to 0.1 mg. nitrogen.

#### 2.4. Enzyme assays.

##### 2.4.1. Nase

Procedure (1): The method depending on the measurement of the ultraviolet absorption of the acid soluble products is based on the procedure described by Lehman, Bessman, Simms & Kornberg (1958). The assay medium containing 300 µg. denatured DNA, 25 µmoles imidazole - HCl buffer pH 6.8, 0.25 µmoles  $MgCl_2$ , 5 µmoles 2-mercaptoethanol in a total volume of 0.4 ml. in a 15 ml. conical centrifuge tube was placed in crushed ice. The enzyme solution was added in 0.1 ml. containing approximately 0.6 units (see this chapter section 2.4.8.). The reaction was started by transferring the tubes rapidly from crushed ice to a shaking water bath at 37°. The normal incubation time was 30 min. at which time the tubes were removed and cooled in crushed ice to stop the reaction. Then 0.3 ml. bovine serum albumin solution (2mg./ml.) was added to act as a carrier and after mixing 1.5 ml. ice cold 1N perchloric acid. After mixing again, the tubes were allowed to stand for 10 min. at 0° before the addition of 0.9 ml. ice cold water. The precipitate was removed by centrifugation at 2° at 1000 x g for 15 min. and the extinction at 260 mµ of the supernatant liquid was



measured. The amount of DNA rendered acid-soluble during the incubation was calculated by assuming a molar extinction coefficient at 260 mμ in acid of 10,200 for the acid-soluble oligonucleotides. This value is the average of the molar extinction coefficients in acid at 260 mμ of the 5'-monophosphates of dAdo, dCyd, dGua and dThd (Burton, 1959) and is regarded as a close approximation to the true value for the acid-soluble DNA fragments, since oligonucleotides do not show hyperchromic effects at 260 mμ on hydrolysis when extinction values are measured at pH 2 (Staehelin, 1961)

Procedure (2) is basically similar to (1) except that the assay medium contained the equivalent for 6 assays and bovine serum albumin was added to the assay prior to incubation. This procedure was used to study the effect of substrate concentration on the rate of the reaction, and therefore  $MgCl_2$  was added to the substrate to give 0.83 μmoles  $MgCl_2$ /mg. DNA so that the ratio of  $Mg^{2+}$ : DNA was constant. The assay medium contained 4.5 mg. bovine serum albumin, 150 μmoles imidazole-HCl buffer pH 6.8, 30 μmoles 2-mercaptoethanol and varying amounts of the substrate. The volume was made up to 2.4 ml. with water. This solution was preincubated in a shaking water bath at 37° for 2 min. The enzyme solution containing approximately 3.6 units in 0.6 ml. was added and after thorough mixing a 0.5 ml.



sample was removed and pipetted into 1.5 ml ice cold 1N perchloric acid at zero time. Further 0.5 ml. samples were removed at specific time intervals in a similar manner. After standing for 10 min. 1.2. ml. ice cold water was added to each of the precipitated fractions, and these were centrifuged as before. The extinction at 260 m $\mu$  of the supernatant liquid was measured.

Unless otherwise stated procedure (1) was employed.

#### 2.4.2. DNase acting on native DNA

The activity of the enzyme towards native DNA was measured by procedure (1) above except that the denatured DNA was replaced by 300  $\mu$ g native DNA. Where native DNA was employed as the substrate, its presence is specified, otherwise reference to DNase activity refers to the activity towards denatured DNA.

#### 2.4.3. RNase

The activity of the enzyme towards RNA was measured by procedure (1) above except that the denatured DNA was replaced by 300  $\mu$ g RNA. The corresponding molar extinction coefficient is 10,600.

#### 2.4.4. DNase II

The method of estimation of DNase II was as procedure (1) above except that the assay medium differed in a number of respects. For DNase II the assay medium contained 300  $\mu$ g.



native DNA, 25  $\mu$ moles acetate buffer pH 4.7 and 100  $\mu$ moles KCl in a total volume of 0.4 ml.

#### 2.4.5. Phosphomonoesterase

The assay medium contained 2  $\mu$ moles p-nitrophenyl phosphate, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 0.25  $\mu$ moles  $\text{MgCl}_2$  and 5  $\mu$ moles 2-mercaptoethanol in a volume of 0.4 ml. The enzyme solution was added in a volume of 0.1 ml. and the reaction was started by transferring the tubes from crushed ice to a shaking water bath at 37°. The reaction was stopped by cooling the tubes in crushed ice followed by the addition of 2.5 ml. 0.1N NaOH. The extinction at 400 m $\mu$  was measured. The molar extinction coefficient of p-nitrophenol under these conditions is 12,000 (Razzell & Khorana, 1959).

#### 2.4.6. Phosphodiesterase I

Phosphodiesterase I is the name given to the enzyme that produces only 5'-mononucleotides on hydrolysis of a polynucleotide at an alkaline pH. The assay was as for phosphomonoesterase except that p-nitrophenyl phosphate was replaced by 2  $\mu$ moles p-nitrophenyl-pT.

#### 2.4.7. Phosphodiesterase II

Phosphodiesterase II acts in a complementary way to phosphodiesterase I producing only 3'-mononucleotides at a slightly acid pH. The assay was as for phosphomonoesterase



except that p-nitrophenyl phosphate was replaced by 2  $\mu$ moles Tp-nitrophenyl. The product of hydrolysis was p-nitrophenol in these last two assays, and therefore the molar extinction coefficient is 12,000.

#### 2.4.8. Enzyme units.

In all experiments on nucleases, and phosphodiesterases and phosphomonoesterases 1 unit of activity is defined as the amount of enzyme which releases 1  $\mu$ mole of nucleotide per hr. The specific activity is expressed as units per mg. protein. The same unit has been applied to all activities measured, but it should be pointed out that only with phosphomonoesterase and exonuclease activities will the value obtained be directly related to the number of bonds hydrolysed. With endonucleases the quantity of nucleotide rendered acid-soluble will only be indirectly related to the number of phosphodiester bonds broken. The factor necessary to convert the number of  $\mu$ moles acid-soluble nucleotides produced to the number of bonds hydrolysed is unknown.

#### 2.5. Preparation of mitochondria

Male albino rats from the department colony weighing between 180-220 g. were given water but no food overnight. The rats were anaesthetised with ether, then killed by exsanguination. Bleeding was allowed to continue for 30 sec. before the livers were excised and immersed in crushed ice.



Subsequent operations were carried out at  $0-4^{\circ}$ . The livers were weighed and cut into small pieces before homogenising in 8 vol. 0.25 M sucrose in a cooled Potter-type homogeniser. After the initial suspension of the tissues, about five passes of the pestle were found to give an homogenate relatively free of whole cells, as judged by microscopic examination of a smear of the suspension stained with 1% crystal violet in 0.1M citric acid. The homogenate was centrifuged at  $600 \times g$  for 10 min. at  $0^{\circ}$ . The supernatant liquid, the cytoplasmic fraction, was decanted from the precipitate which contained the nuclei and cellular debris. Assays of nuclease activities in the nuclei were performed on a suspension of nuclei in 0.25M sucrose subjected to ultrasonic vibrations at 20Kc. and 50W. for 30 sec. The cytoplasmic fraction was centrifuged at  $14,000 \times g$  for 10 min. The supernatant liquid was discarded and the precipitate consisting largely of mitochondria and lysosomes was used immediately. The nuclease activities in this precipitate were assayed by suspending the material in 0.25M sucrose and disrupting the particles by ultrasonic vibrations at 20Kc. and 50W.

The mitochondria were initially suspended in a small volume of 0.01M tris-HCl buffer pH 7.5 and 0.1% (w/v) with respect to triton-X-100 by gentle homogenisation, and were dispersed into a volume of 0.01M tris-HCl buffer pH 7.5 and 0.1% (w/v) with respect to triton-X-100 1.25 times the



volume of the original homogenate. The solution was briefly homogenised at very slow speed.

## 2.6. Enzyme fractionation techniques.

All operations were carried out at  $0^{\circ}$  except where stated otherwise.

### 2.6.1. Ammonium sulphate fractionation.

Saturated ammonium sulphate was prepared at room temperature (approx.  $20^{\circ}$ ) and was adjusted to pH 7.5 when completely equilibrated. To the solubilised mitochondria containing approximately 3 mg. protein/ml. saturated ammonium sulphate was added slowly with stirring to give the required degree of saturation. Magnetic stirring was used to prevent frothing. Stirring was continued for 20 min. after the complete addition of saturated ammonium sulphate. The solution was centrifuged at  $14,000 \times g$  for 20 min. The precipitate was dissolved in the minimum volume of 0.01M tris-HCl buffer pH 7.5. The above procedure was repeated on the supernatant liquid to give the required fractions. The dissolved precipitates were dialysed overnight in 0.01M tris-HCl buffer pH 7.5 at  $0^{\circ}$  before being assayed for nuclease activity and protein content.

### 2.6.2. Acetone fractionation

The ammonium sulphate content of the active fraction



obtained after fractionation with ammonium sulphate was determined (this chapter, section 2.3.3.) and was adjusted to 80 mg./ml. This procedure obviated the necessity of dialysis by allowing the acetone fractionation to be performed at a known salt concentration. The ammonium sulphate fraction usually contained approximately 10 mg. protein/ml. For every 10 ml. of the solution 2 ml. 1M tris-HCl buffer pH 6.5 were added, and this solution was immersed in a glycol bath at  $-15^{\circ}$ . Acetone, previously cooled to  $-15^{\circ}$ , was added immediately to give the required concentration, measured v/v, with rapid stirring. The solution was allowed to stand for 5 min. before centrifugation at  $-15^{\circ}$  at  $700 \times g$  for 15 min. The precipitate obtained after carefully draining off the supernatant liquid was dissolved in the minimum volume of 0.01M tris-HCl buffer pH 7.5. The supernatant liquid was further fractionated according to the above procedure, and the dissolved precipitates were dialysed overnight in 0.01M tris-HCl buffer pH 7.5 before being assayed for nuclease activity and protein.

#### 2.6.3. The preparation and use of DEAE-cellulose.

DEAE-cellulose was prepared for use by preliminary steeping in three times its own volume of 0.5N NaOH and 0.5M with respect to NaCl. The suspension was stirred vigorously for 5 min. and then allowed to settle for 10 min.



The supernatant liquid was removed by suction together with any fine particles that had remained in suspension. The alkali washing was repeated until the supernatant liquid was colourless. The cellulose was then suspended in water. Decantation and resuspension in water was continued until the supernatant liquid was relatively free of fine particles, allowing 10 min. for the cellulose to settle. The suspension was transferred to a Buchner funnel and washed with water until free of alkali. The cellulose was resuspended in 0.1N HCl, which was immediately removed by filtration on a Buchner funnel with suction. The cellulose was washed with water until free of acid. The cellulose was again suspended in 0.5N NaOH and 0.5M with respect to NaCl, and after standing for 10 min. was collected by filtration on a Buchner funnel. Washing with water was continued to remove the alkali. Finally the washed cellulose was suspended in a buffer whose concentration was 5 times that of the required buffer, and it was allowed to equilibrate overnight. The pH was then adjusted to the desired value if necessary. Various sizes of columns were used, but all were packed with the application of pressure (approx. 15 cm. Hg.) to ensure an evenly packed column. With such a pressure it was not found necessary to stabilise the top surface of the column. The column was washed with approximately 10 bed volumes of



the starting buffer. The pH of the effluent was checked to ensure that it was the same as that of the eluting liquid. The column was transferred at this stage to the cold room at 4°. Elution of the enzyme in all the experiments to be described was achieved with the starting buffer. The flow rate was adjusted to give approximately 40 ml./cm<sup>2</sup>./hr. by adjusting the height of a reservoir connected to the column. The volume of the fractions collected varied with the size of the column used. The fractions containing material absorbing in the ultraviolet at 280 mμ were assayed for nuclease activity and protein. Each column was used only once. After use, the cellulose was removed from the column and stored under water saturated with toluene to inhibit bacterial growth. When a sufficient quantity had been collected, the whole batch was regenerated by the procedure described above.

CM-cellulose, CM-Sephadex and DEAE-Sephadex were prepared in a similar manner. However no pressure was applied when packing columns with these materials.

#### 2.6.4. The use of gel filtration

The uncharged dextran gels Sephadex G-75 and G-200 were used as molecular sieves allowing the separation of protein molecules according to molecular size and shape.

The weighed amount of dry dextran powder was suspended



in excess of 0.1% (w/v) NaCl with stirring to avoid clumping and the gel was allowed to equilibrate for 24 hr. After equilibration the fine particles were removed by resuspending the gel and then allowing the gel to settle. When a sharp boundary became visible, the supernatant liquid was removed by suction and this treatment was repeated until the supernatant liquid was clear. The gel was then suspended in the starting buffer 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol.

Careful packing of the column was essential to obtain efficient separation of protein molecules. The packing was performed at room temperature. Two sizes of column were used, 1.7 cm. diameter by 110 cm. and 2.3 cm. diameter by 110 cm. The column was fitted with a circular disc of sintered polythene above the outlet tube, and the disc was covered with a thin layer of fine glass beads which supported the gel and prevented the blocking of the sintered polythene. The column was mounted vertically and was filled completely with 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol. A large filter funnel was fitted to the top of the column and the gel suspension was poured into the funnel. The suspension was stirred throughout the process of packing. When 4-5cm. of gel had settled, the outlet tube was opened. It was found with Sephadex G-75 that the column gave better



separation if it was packed as tightly as possible, consequently the outlet tube was opened to give the maximum flow rate. The resulting flow rates from the prepared columns were always higher than that required for separation of protein molecules. With Sephadex G-200 the flow rate was reduced to a very low rate if the gel was packed too tightly. During the packing therefore the flow rate was adjusted to be approximately that used in the subsequent separation. The level of the gel bed was horizontal at all times during the packing of the column and the packing was continued until the bed height was 2-3 cm. above that required. The column was placed in a cold room at 4° and connected to a reservoir containing 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol. Washing of the column was continued for 2 days. With Sephadex G-75 the reservoir was held approximately 2 m. above the base of the column, but with Sephadex G-200 the height of the reservoir was between 10-30 cm. above the base of the column. After washing, the height of the bed was adjusted to the required level and the gel was then allowed to settle. If the bed surface was not horizontal, the top of the column was stirred and then allowed to resettle. This process was repeated until the bed surface was horizontal. The surface of the gel was stabilised



by stretching a piece of nylon mesh over the rim of a piece of glass tubing that fitted tightly into the column and inserting the mesh into the column so that it rested on the surface of the gel. Before use the void volume of the column was determined by measuring the elution volume, that is the volume of buffer required to move material that is completely excluded from the gel particles from the top to the bottom of the column. A dyed dextran, Blue Dextran 2000, with a molecular weight of  $2 \times 10^6$  was used as a 0.5% (w/v) solution in 0.2M sucrose. Before addition to the column the outlet tube was closed, and 1 ml. dextran solution was introduced below the buffer onto the surface of the gel. with very little mixing. The column was connected to a reservoir and the outlet tube was opened to give a flow rate of 6 ml./cm.<sup>2</sup>/hr. The effluent was collected in 3 ml. fractions and the extinction at 625 mμ was measured. The fraction having the largest extinction was taken as corresponding to the void volume. After 2-3 runs Sephadex G-200 columns became very slow as the result of packing and clogging. The columns were therefore repacked after the gel had been washed. Columns packed with Sephadex G-75 were more stable. Clogging occurred at the bed surface and thus was removed and replaced with fresh gel.



### 3. Results

#### 3.1. The partial purification of a nuclease from rat liver.

Beaufray et al. (1959) showed that the alkaline DNase (acting on native DNA) of rat liver was largely located in the mitochondria. However other subcellular fractions contained activity, and only 50% of the total activity in the cell occurred in mitochondria. The work of Burdon (1963) showed that alkaline DNase acting on denatured DNA had a similar distribution. The technique of Beaufray et al. (1959) of separation of subcellular particles by differential centrifugation was applied to rat liver, but the results obtained indicated that little or no separation of mitochondria from lysosomes was obtained. Since it seemed likely that even poorer separations would be achieved when working on a larger scale it was decided to use a fraction that would contain all the mitochondria and probably the majority of the lysosomes. To verify that such a procedure would give a reasonable yield of the enzyme a partial subcellular fractionation was performed on the liver of a male rat. The method and conditions were those described in this chapter section 2.5. The precipitated nuclei and mitochondria were resuspended in 0.25M sucrose, and each fraction, that is the whole homogenate, the nuclei, the cytoplasmic fraction with the nuclei removed and the



mitochondria were subjected to ultrasonic vibrations at 20Kc. and 50W. for 30 sec. The fractions were then assayed for DNase and protein (Table I.) The experiment showed that the mitochondrial fraction contained 88% of the activity present in the whole homogenate and 98% of the activity in the cytoplasm after removal of the nuclei. Such a mitochondrial fraction thus provided a useful source of the enzyme. Before further fractionation of the enzyme was attempted, a method was sought to bring all the enzyme into solution. A detergent triton-X-100 was considered, for it was thought that such a material would perhaps cause the disaggregation of molecular complexes involving lipoproteins and thus bring about the release of the enzyme if it were bound to a membrane. The use of this detergent resulted in no loss of nuclease activity and gave a solution relatively free of particles.

The mitochondrial solution was fractionated according to the procedures of Burdon et al. (1964) These procedures involved acid precipitation at pH 4.5 of an aqueous extract, followed by ammonium sulphate fractionation which consisted of collecting the precipitate obtained between 40 - 60% saturation. Finally the preparation was fractionated with acetone, the precipitate obtained between 36.9 - 45.4% acetone (w/v) being taken.



**TABLE 1.**

**The intracellular location of the liver DNase.**

**The subcellular fractions were suspended in 0.25M sucrose and subjected to ultrasonic vibrations at 20 Kc. and 50 W. for 30 sec. before being assayed for DNase and protein.**



**TABLE 1**

Fraction	Volume ml.	Activity units/ml.	Total No. of Units	Protein mg/ml.	Total amount of protein	Specific activity units/mg.	% Recovery
Homogenate	70	5.9	413	12.1	850	0.48	100
Nuclei	30	1.4	43	8.3	250	0.17	10.4
Cytoplasm	70	5.3	370	8.6	600	0.62	90
Mitochondria	140	2.6	360	0.8	108	3.3	88



It was found that acid precipitation at pH 4.5 gave a very poor yield of the enzyme, and this step was therefore discarded. Ammonium sulphate precipitation of the mitochondrial solution resulted in the enzyme being distributed between 20-40% and 40-60% saturation (Table 2). The experiment was therefore repeated using the fractions obtained at 0-35%, 35-55% and 55-70. saturation. It was found that more than 80% of the enzyme was located in the fraction precipitating between 35-55% saturation with a 2-fold increase in the specific activity (Table 2). This fraction was used for the subsequent acetone fractionation. It was found that the enzyme was precipitated between 37.8-45.5% acetone (v/v, assuming the volumes to be additive) giving a 2-fold increase in the specific activity and a 50% yield of units of activity. The enzyme had therefore been precipitated over the same range of acetone concentration as before (Table 3).

The use of ion-exchange cellulose was limited by the earlier findings that where adsorption of the enzyme to charged dextran gels or charged cellulose occurred, recovery of the active enzyme was very low. Conditions were therefore sought where the enzyme was not adsorbed, but as much of the inactive protein as possible was adsorbed. Other limitations were found in the conditions that could



TABLE 2.

Ammonium sulphate fractionation of the solubilised mitochondria.

The ammonium sulphate precipitated fractions were dissolved in the minimum volume of 0.01M tris-HCl pH 7.5 and dialysed against the same buffer overnight. The fractions were assayed for DHase and protein.



Fraction	Volume ml.	Units per ml.	Total No. of Units	Total amount of protein mg.	% of Total protein	% of Total Units	Specific Activity units/mg.
<u>Expt. 1</u>							
Triton-X-100	150	4.5	672	445	100	100	1.5
AS 0-20	10	0.06	0.6	10	2	0.1	0.1
AS 20-40	35	6.1	213	154	34.2	31.7	1.4
AS 40-60	28	12.8	360	142	32	54	2.5
AS 60-80	15	0.34	5.1	39	8	0.7	0.1
<u>Expt. 2</u>							
Triton-X-100	150	8.1	1210	536	100	100	2.3
AS 0-35	35	0.5	17.6	133	24.8	1.4	0.7
AS 35-55	25	40.5	1010	213	40.0	84	4.7
AS 55-70	11	0.15	1.6	40.4	7.5	0.1	0.4



TABLE 3.

Acetone fractionation of the fraction precipitating between 35-55% saturation with ammonium sulphate.

The precipitates were dissolved in the minimum volume of 0.01M tris-HCl pH 7.5 and dialysed against the same buffer overnight. The fractions were then assayed for DNase and protein.



TABLE 3

Fraction	Volume ml.	Units per ml.	Total No. of Units	Total amount of protein mg.	% of Total protein	% of Total Units	Specific Activity units/mg.
AS 35-55	20	38	790	170	100	100	4.7
Acetone concn. 0-33.3%	32	2.0	63.5	72	42	8	0.9
33.3-37.8%	30	3.6	109	69	40	13.8	1.6
37.8-45.5%	15	24.6	370	41.4	24	49.5	8.9
45.5-52.0%	10	0.35	3.5	2	1.2	0.4	1.8



be used. The stability of the enzyme was lower at pHs below 6.0 and above 8.5. At ionic strengths below 0.01M, considerable adsorption of the enzyme occurred. Therefore the optimal conditions within these limitations were sought. DEAE-cellulose was prepared and equilibrated in 0.01M imidazole-HCl buffer pH 6.8, 0.01M tris-HCl buffer pH 7.5, 0.1M tris-HCl buffer pH 7.5 and 0.01M tris-HCl buffer pH 8.0. A column 1 cm. diameter by 20 cm. was packed under pressure and washed with the buffer in which it was equilibrated until the pH of the effluent was the same as the eluent. After equilibration of the column at 4° a sample of the dialysed acetone fraction was applied after removal of the supernatant buffer. When the solution had passed into the cellulose the surface was washed with the same buffer and the column was connected to a reservoir containing more of the buffer. The effluent was collected in 2 ml. fractions. Those fractions showing ultra-violet absorption at 280 mμ were assayed for DNase activity and protein. The experiment was repeated with the four different buffers and the results are given in Table 4. The values for the effluent fractions were obtained by summation of all those fractions showing activity. The results showed that increasing the ionic strength increased the yield of the enzyme, but at the same time reduced the adsorption of inactive protein, so that there was a reduction in the purification. Secondly,



TABLE 4.

The effect of ionic strength and pH on the adsorption of the liver nuclease to DEAE-cellulose.

Four columns, 1 x 20 cm., were equilibrated to 0.01M imidazole-HCl buffer pH 6.8, 0.01M tris-HCl buffer pH 7.5, 0.1M tris-HCl buffer pH 7.5, and 0.01M tris-HCl buffer pH 8.0. 0.7 ml. of the 36.9-46.9% acetone fraction containing 5.0 mg. protein and 32 units was applied to each column. The eluting buffer was the same as that used for the equilibration of the column. The effluent was collected in 2.0 ml. fractions. The fractions containing ultraviolet absorbing material at 280 mμ were assayed for protein and DNase, and the values given in the table were obtained by summation of those fractions showing activity.



**TABLE 4**

Eluting Buffer	Volume of Total Fraction ml.	Units per ml.	Total No. of Units	Total amount of protein mg.	Specific Activity units/mg.	Yield %	Purification
Acetone 36.9-46.9%	0.7	45	32	5	6.5	100	1
Imidazole 0.01M pH 6.8	4.0	1.9	7.8	0.79	10.0	24	1.54
Tris 0.01M pH 7.5	6.5	2.9	18.8	0.51	37.0	59	5.2
Tris 0.1M pH 7.5	6.5	3.9	25.2	1.48	17.0	79	2.6
Tris 0.01M pH 8.0	4.5	6.0	27	0.37	74.0	85	11.4



raising the pH increased the adsorption of inactive protein, while reducing the adsorption of the enzyme. Therefore 0.01M tris-HCl buffer pH 8.0 provided conditions close to the optimal for the use of DEAE-cellulose. It was also found that increasing the ratio of protein/DEAE-cellulose beyond approximately 1 mg. protein/3 ml. packed bed volume resulted in a reduction of the purification, though an increased yield was obtained.

An increase in the scale of the operations to a column with a bed volume of 700 ml. (4.7 cm. diameter by 40 cm.) and with a bed volume of 1200 ml. (5.5 cm. diameter by 50 cm.) gave similar results, though it was noted that a reduction in the yield from 80% to 60% was usually obtained.

The use of gel filtration as a means of purifying the enzyme was examined. To apply this technique it was first necessary to concentrate the enzyme into a small volume. The effluent from a DEAE-cellulose column was therefore concentrated by treating the enzyme with saturated ammonium sulphate solution to 70% saturation. The precipitate was collected by centrifugation at 14,000 x g for 20 min. and was dissolved in 1 ml. 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol. The use of 2-mercaptoethanol had been indicated by the finding that it increased the stability of the enzyme. The gel was also equilibrated with 0.01M imidazole-HCl buffer pH 6.8 and



0.005M with respect to 2-mercaptoethanol. A column (2.3 cm diameter by 110 cm.) was packed with Sephadex G-200. The concentrated enzyme was applied to the bed surface. It was unnecessary to add sucrose to the concentrated solution as the protein and ammonium sulphate raised the density sufficiently to ensure that no mixing occurred during the application. The eluting buffer was 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol, the flow rate was 3 ml./hr. and 10 ml fractions were collected. Each fraction was assayed for DNase, RNase and protein (Fig.3). It was necessary to dialyse samples that were to be used for protein estimation for it was observed that 2-mercaptoethanol interfered with the estimation of the protein concentration. The enzyme was eluted with a minor peak of protein along with a peak of RNase coinciding with the peak of DNase. This result was contrary to that obtained when the enzyme had been prepared from an aqueous extract of the whole tissue and applied to a Sephadex G-200 column. In that experiment the enzyme was eluted with the void volume. The present procedure had thus presumably resulted in the disaggregation of the complex that had been purified by the earlier method. The position of the eluted enzyme indicated that better separation would be obtained using Sephadex G-75. The experiment was therefore repeated



Figure 3.

The separation of the DEAE-cellulose fraction on Sephadex G-200.

The precipitated DEAE-cellulose fraction was dissolved in 1.3 ml. 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol, containing 36 mg. protein and 750 units. The eluting buffer was 0.01M imidazole-HCl pH 6.8 and 0.005M with respect to 2-mercaptoethanol. The column was 2.3 x 108 cm., the flow rate was 2.5 ml./hr. and 10 ml. fractions were collected. Each fraction was assayed for DNase, RNase and protein.

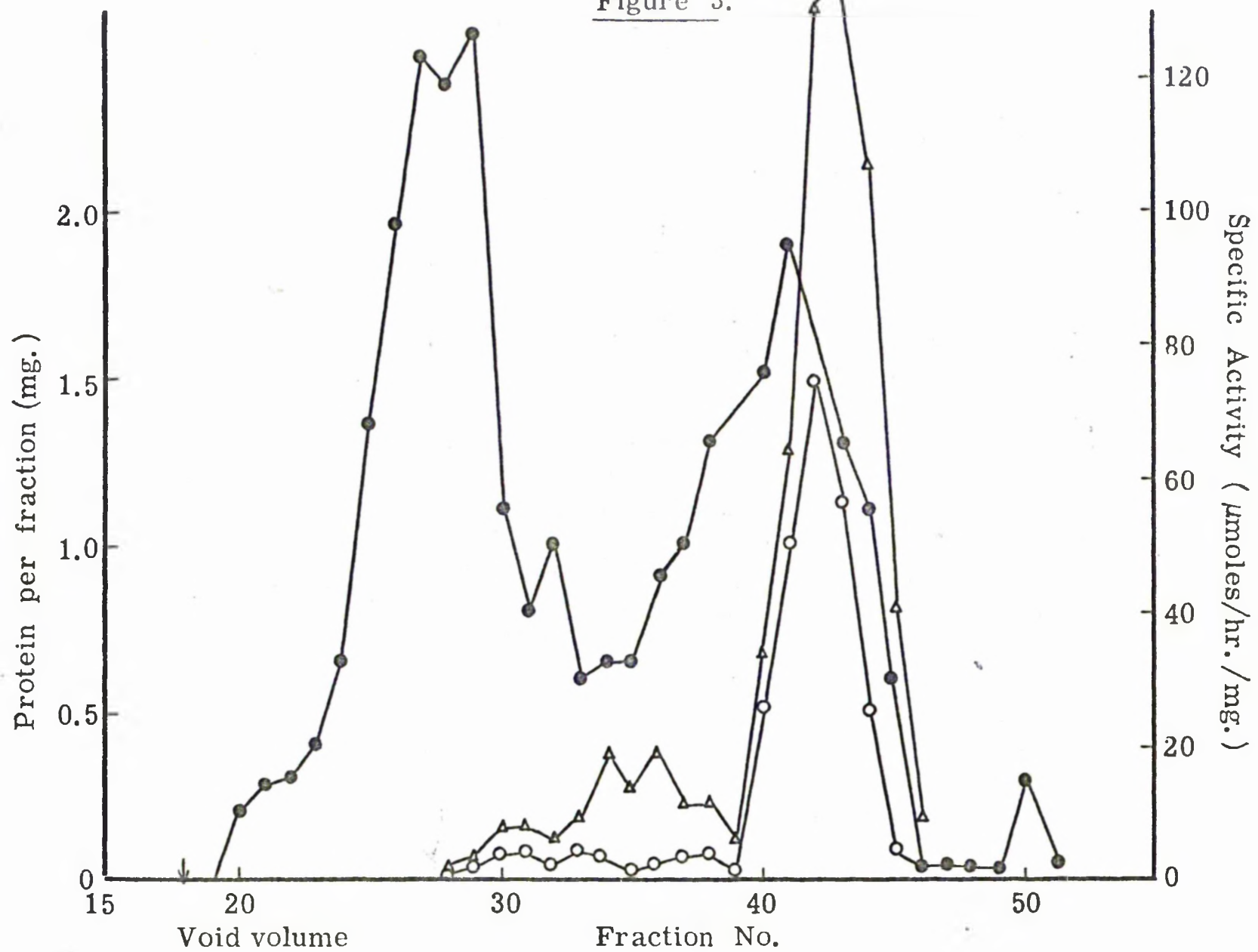
Protein —●—

DNase —○—

RNase —△—



Figure 3.





using a column (1.7 cm. diameter by 110 cm.) of Sephadex G-75 equilibrated with the same buffer. The concentrated enzyme solution was applied as before. The flow rate was 8 ml./hr. and 3 ml. fractions were collected. Each fraction was assayed for DNase, DNase with native DNA as a substrate, RNase and protein. The peak of DNase activity was separated from the major protein peak (Fig.4). Also coinciding with the DNase peak was the activity towards native DNA and RNA. Since the specific activity of the DNase varies through the peak and there is no isolated peak of protein corresponding to the peak of activity, the enzyme would appear not to be homogeneous at this stage. From this experiment fractions 32-35 were combined and assayed for DNase II, phosphomonoesterase, phosphodiesterase I and II. 0.1 ml. of the undiluted enzyme solution was used, and incubation was for 4 hr. so that any activity might show more clearly. Under these conditions the activities did not amount to more than 0.1% of the total amount of DNase present (Table 5). These results indicated that at this level of purification studies on the properties and specificity of the liver DNase could usefully be carried out since contamination with DNase II, phosphomonoesterase, phosphodiesterase I and II was negligible. The additional observation that RNase activity was associated closely with



Figure 4.

The separation of the DEAE-cellulose fraction on Sephadex G-75.

The precipitated DEAE-cellulose fraction was dissolved in 1 ml. 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol, containing 4.5 mg. protein and 405 units. The column, 1.7 x 106 cm., was equilibrated with 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol, and was eluted with the same buffer. The flow rate was 8 ml./hr. and 3 ml. fractions were collected. Each fraction was assayed for DNase, DNase acting on native DNA, RNase and protein.

Protein	—●—
DNase	—○—
DNase acting on native DNA	—□—
RNase	—△—



Figure 4.

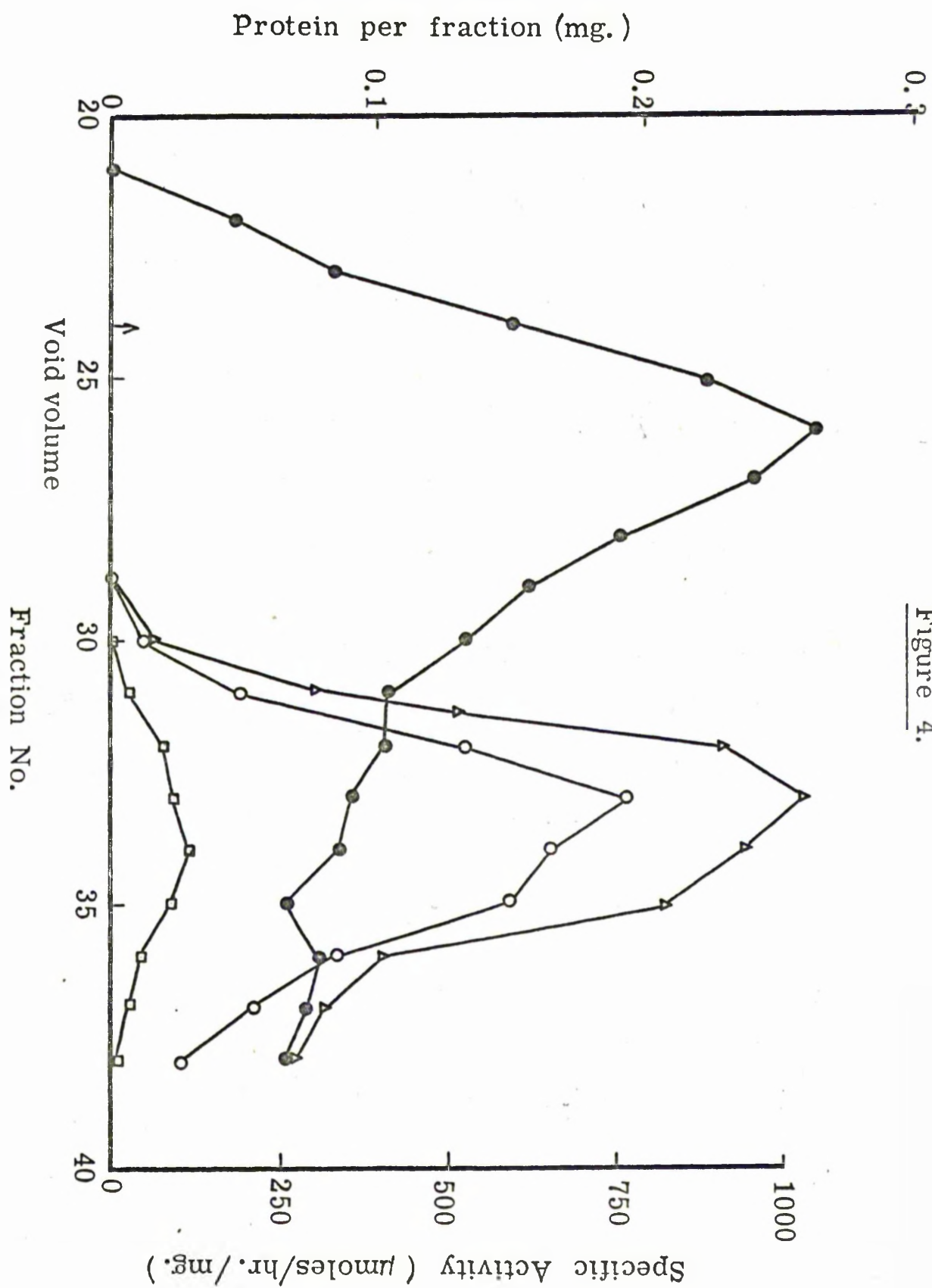




TABLE 5

The contaminating activities in the purified liver nuclease (Sephadex G-75 effluent).

Activity	Total No. of units in fractions 32-35
DNase	206
DNase acting on native DNA	31.1
RNase	345
DNase II	0.1
Phosphatase	0.0
Phosphodiesterase I	0.02
Phosphodiesterase II	0.05



the DNase fractions obtained from both Sephadex G-75 and G-200 indicated that this activity together with its activity towards native DNA might be an inherent property of the DNase. The activities of the enzyme towards RNA and native DNA were therefore examined in the various purification procedures, and Table 6 shows the ratios of these activities to DNase. The ratios are relatively constant, the main discrepancies being in the homogenate and the final fractions. The values of these ratios for the homogenate are probably not too meaningful since they represent net results of many concurrent reactions. While those shown for the final fraction are rather different from the ratios obtained at earlier stages in the purification it should be mentioned that values closer to the average have been obtained in other experiments.

The activity towards native DNA was examined more closely. There are two enzymes, exonuclease I from E. coli (Lehman & Nussbaum, 1964) and an endonuclease from lamb brain (Healey et al., 1963), which showed in the initial stages of purification a partial preference for denatured DNA. After extensive purification these two enzymes possessed an absolute requirement for denatured DNA. The endonuclease from lamb brain resembles the liver DNase in a number of other respects which might lead one to suggest



**TABLE 6**

The ratios of the activities of various enzyme fractions towards (i) denatured and native DNA (ii) denatured DNA and RNA.

Fraction	DNase/DNase <sub>N</sub> <sup>*</sup>	RNase/DNase
Homogenate	13.7	3.3
Triton-X-100	2.3	2.8
AS 35-55%	2.8	3.6
Acetone 36.9-46.9%	3.2	2.3
DEAE-cellulose	2.2	3.4
AS 0-70%	1.9	4.0
Sephadex G-75 (fractions 32-35)	7.0	1.5

\* DNase<sub>N</sub> denotes DNase acting on native DNA.



that they would be identical. Therefore the activity towards native DNA in the liver DNase may reflect the presence of some denatured regions in the preparation of native DNA. To check this point commercial calf thymus DNA was used, and additional support was sought by using sRNA that had been shown to have the properties of a double helix by studies in ultracentrifugation and by measurements of its hyperchromicity on heating. The results (Table 7.) would seem to indicate that the ability to hydrolyse a double helical structure is an intrinsic property of the nuclease.

The procedures described thus provide a method of purifying the liver DNase to a stage where it is relatively free from DNase II, phosphomonoesterase, phosphodiesterase I and II.

### 3.2. Procedure adopted for the purification of the nuclease from rat liver.

All operations were carried out at  $0-4^{\circ}$  except where stated otherwise.

#### 3.2.1. Preparation of mitochondria.

Mitochondria were collected from the livers of 10 male rats as previously described (this chapter, section 2.5.) The livers weighed about 70g. wet weight. The



**TABLE 7**

The effect of the secondary structure of the substrate on the activity of the purified liver nuclease (Sephadex G-75 effluent).

Substrate	nmoles/assay/30 min.	Ratio S/D*
Denatured DNA	147	6.6
Native DNA	22.5	
RNA	370	4.6
sRNA	81	

\*Ratio S/D denotes the ratio of the activities towards denatured or single stranded substrates and native or double stranded substrates.

Native DNA was calf thymus DNA, purchased from the Sigma Chemical Co., and denatured DNA was heat denatured calf thymus DNA. RNA was highly polymerised yeast RNA purchased from B.D.H. sRNA was prepared from yeast cells by the method of Bell et al. (1964).



mitochondrial solution contained usually 3-4 mg. protein/ml. and its volume was of the order of 600 ml. (designated Fraction I).

### 3.2.2. Fractionation with ammonium sulphate.

Ammonium sulphate solution, saturated at room temperature and adjusted to pH 7.5 with 2N ammonium hydroxide was used. The required volume of saturated ammonium sulphate to bring the mitochondrial solution to 35% saturation was added slowly with continuous stirring. After all the ammonium sulphate had been added the solution was stirred for a further 20 min. It was then centrifuged at 14,000 x g for 20 min. The precipitate was occasionally found to form a pellet floating on the surface as well as a pellet in the bottom of the solution. The supernatant liquid was therefore strained through 2 layers of muslin which retained any precipitate floating in the solution. To this solution a further volume of saturated ammonium sulphate was added slowly to bring the degree of saturation to 55%. Stirring was continued for 20 min. after completing the addition. The precipitate was collected by centrifugation at 14,000 x g for 20 min. The supernatant liquid was discarded and the centrifuge bottles were inverted and allowed to drain for 1 min. The inside of the bottles were then wiped with absorbent tissue in order to remove as much of the ammonium



sulphate as possible. The precipitate was then dissolved in the minimum volume of 0.01M tris-HCl buffer pH 7.5, normally 50-70 ml. (designated Fraction II). The amount of ammonium sulphate present was estimated using Nessler's reagent. 0.1 ml. of the protein solution was diluted to 50 ml. with water. 2 ml. were pipetted into 5 ml. water, and 1 ml. Nessler's reagent was added. The solution was mixed and allowed to stand for a few minutes before the extinction was read at 490 mμ. Having previously standardised the method it was thus possible to determine the quantity of ammonium sulphate present in approximately 10 min. The concentration was adjusted to 80 mg./ml. by dilution or by addition of solid ammonium sulphate.

### 3.2.3. Fractionation with acetone.

200 ml. acetone had previously been immersed in a glycol bath at  $-15^{\circ}$ . The ammonium sulphate fraction normally contained approximately 10 mg. protein/ml. To the solution 2 ml. 1M tris-HCl buffer pH 6.5 was added for every 10 ml. of the solution. The solution was then immersed in the glycol bath at  $-15^{\circ}$ , and the measured volume of pre-cooled acetone to bring the solution to 36.9% acetone (v/v) was added immediately with stirring. This concentration of acetone corresponds to 7 ml. acetone for every 10 ml. of the original solution. Stirring was continued for 5 min. The solution



was then centrifuged at  $-15^{\circ}$  at 700 x g for 15 min. The supernatant liquid was decanted into a conical flask which had previously been immersed in the glycol bath at  $-15^{\circ}$ . To it was added the required volume of pre-cooled acetone to bring the concentration to 46.9% (v/v), that is a further 3.5 ml. acetone for every 10 ml. of the original solution. The solution was stirred for 5 min. after which it was centrifuged at 700 x g for 15 min. The precipitate formed a protein pellet situated between two liquid phases. When no such separation of the liquid phase occurred, it was invariably found that the preparation was relatively inactive. The liquid was drained away, and the pellets were dissolved in a minimum volume of 0.01M tris-HCl buffer pH 7.5 normally between 10-20 ml., and the solution was dialysed against 7 litres 0.01M tris-HCl buffer pH 7.5 for approximately 5 hr. The final product was then frozen and stored at  $-20^{\circ}$  (designated Fraction III).

#### 3.2.4. Fractionation on DEAE-cellulose

DEAE-cellulose was prepared as previously described (this chapter, section 2.6.3.) and was equilibrated in 0.05M tris-HCl buffer pH 8.0. The column (4.7 cm. diameter by 40 cm.) was packed under 15 cm. Hg. pressure at room temperature giving a bed volume of 700 ml. The maximum capacity of the column was between 200-300 mg. protein. The



normal yield of protein after the acetone fractionation from 10 rats was about 200 mg. The column was washed with about 7 litres 0.01M tris-HCl buffer pH 8.0. With a hydrostatic pressure of approximately 1 m. a flow rate of 700 ml./hr. was obtained. Washing was continued until the pH of the effluent was the same as the eluent. The column was removed to the cold room where it was allowed to cool overnight. With the outlet tap closed, the supernatant liquid was removed and the protein solution was carefully layered on the column. The outlet tap was opened, and the surface of the column was washed with 0.01M tris-HCl buffer pH 8.0 when the protein solution had entered the column. After the washing of the surface approximately 100 ml. 0.01M tris-HCl buffer pH 8.0 was added to the column which was then connected to a reservoir containing the same buffer held 1m. above the surface of the cellulose. Under these conditions a flow rate of 700 ml./hr. could be attained.

From previous experiments it was known that such a column would have a void volume of approximately 500 ml. Thus 400 ml. was collected initially, then 20 ml. fractions were collected. The extinction of these fractions was measured at 280 mμ. Initially these fractions containing protein were assayed for DNase, RNase and protein (Fig.5).



Figure 5.

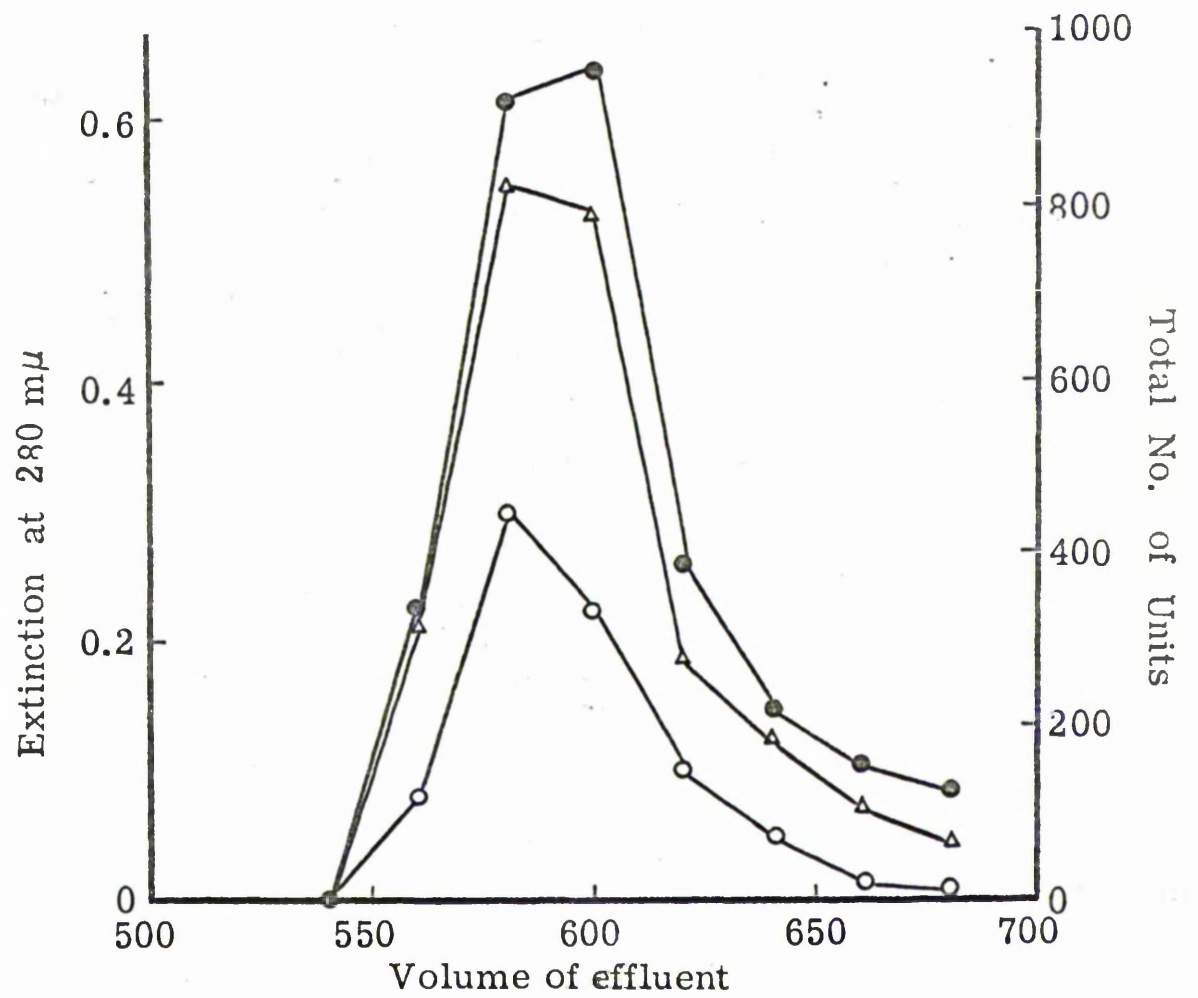
The elution of the acetone fraction from DEAE-cellulose.

The acetone fraction, 19 ml., contained 210 mg. protein, 1880 DNase units and 2960 RNase units. The column, 4.7 x 40 cm., was equilibrated with 0.01M tris-HCl buffer pH8.0, and was eluted with the same buffer. The effluent was collected in 20 ml. fractions after the first 400 ml. The extinction at 280 mμ of each fraction was measured, and each fraction was assayed for DNase and RNase

Protein —●—  
DNase —○—  
RNase —△—



Figure 5.





However this procedure was time consuming and wasteful with respect to enzyme units. The procedure normally adopted was to read the extinction at 280 m $\mu$ , and those fractions with an extinction greater than 50% of the maximum extinction obtained were combined to provide the enzyme solution normally 60-80 ml. for further fractionation (designated Fraction 1V).

### 3.2.5. Fractionation on G-75

Sephadex G-75 was prepared and packed as described in this chapter section 2.6.4. The column, 1.7 cm. diameter x 106 cm., was equilibrated with 0.01M. imidazole-HCl buffer and 0.005M with respect to 2-mercaptoethanol at 0-4<sup>0</sup>. The void volume had previously been determined using blue dextran and found to be 72 ml. The enzyme was concentrated from the DEAE-cellulose effluent by precipitation in 70% saturated ammonium sulphate. The precipitate was collected by centrifugation at 14,000 x g for 20 min. and was dissolved in 1 ml. 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol. This concentrated solution was applied to the bed surface below the supernatant buffer, and the column was connected to a reservoir containing the same buffer. The outlet tap was opened and the flow rate adjusted to 8 ml./hr. by varying the height of the reservoir. The effluent was



collected in 3 ml. fractions. The appropriate fractions were assayed for DNase, RNase and protein. Those fractions containing the enzyme with a specific activity greater than 50% of the maximum value were pooled to give the final enzyme solution (designated Fraction V).

A summary of a purification procedure is given in Table 8. The overall purification of DNase is 715, though it should be noted that the fraction containing the highest specific activity corresponds to 1100 fold increase in the specific activity. The enzyme solution was in general used immediately, but for some experiments was kept for upto a week in 20% glycerol.

### 3.3. Storage of the nuclease.

The work of Burdon (1963) had shown that the nuclease after acetone fractionation could be stored at  $-20^{\circ}$  or  $-70^{\circ}$  for upto a month without loss of activity. However after passage through DEAE-cellulose the stability of the enzyme was considerably reduced. The convenient methods of storage, that is at low temperatures or freeze-drying, resulted in large losses. However the paper by Healey et al. (1963) suggested two methods of storing the enzyme for periods of upto a week without considerable losses. Samples from a DEAE-cellulose affluent were set up in the presente



**TABLE 8**

**The purification of Diase from rat liver**

Fraction	Total Volume mg.	Units per ml.	Total No. of Units	Total amount of protein mg.	Specific Activity units/mg.	Yield %	Purification
Homogenate	500	10	5000	7300	0.69	100	1
Triton-X-100 Fr. I	650	9.6	6250	2200	2.85	120	4.1
AS 35-55% Fr. II	80	44.5	3560	720	4.78	71	7
Acet. 36.9-46.9% Fr. III	20	165	3300	210	15.7	66	23
DEAE-cellulose Fr. IV	80	11.7	940	7.0	134	19	195
AS 0-70%	1	506	506	4.5	112.5	10	163
Sephadex G-75 Fr. V	12	17.1	206	0.44	492	4	715



of 20% glycerol and 0.01M 2-mercaptoethanol and stored in ice. The activity of these solutions was then compared with the activity of a solution of the enzyme in the presence only of the buffer after various time intervals (Table 9). In the presence of the buffer only the enzyme lost DNase activity rapidly, but RNase activity less rapidly. In the presence of 2-mercaptoethanol there was an early loss of DNase after which there was no substantial fall over a period of 3 weeks. In comparison the RNase appeared to be much more stable, and only showed a small loss of activity. In 20% glycerol both activities were stable for upto a week, though after 4 weeks both showed losses. The effect of 2-mercaptoethanol, together with the fact that EDTA showed a stimulatory effect at very low concentrations, suggested that the enzyme required thiol groups. However assays performed on freshly prepared DEAE-cellulose effluent showed no dependency on 2-mercaptoethanol, though after the enzyme solution had been stored in ice for 5, 30, 50, and 120 hr. the assay for both DNase and RNase was stimulated by about 17% (Fig. 6) Preliminary experiments indicated that the enzyme was not inhibited by thiol reagents sodium p-hydroxymercuribenzoate and iodoacetamide at concentrations where thiol enzymes are inhibited.



TABLE 9.

The effect of storage on the activity of the liver nuclease.

Samples, 2 ml., contained 0.02M imidazole-HCl buffer pH 6.8 and 83 ug. protein of fraction IV per ml. The samples were stored in ice in the presence of (i) no further additions (ii) 20% glycerol (iii) 0.01M 2-mercaptoethanol. The samples were assayed for DNase and RNase at zero time and after 3, 6 and 28 days. The results are expressed as a percentage of the zero time values.



**TABLE 9**

<b>Storage Conditions</b>		<b>% of the original activity</b>		
		<b>3 days</b>	<b>6 days</b>	<b>28 days</b>
<b>Buffer only</b>	<b>DNase</b>	<b>3.8</b>	<b>0</b>	<b>4</b>
	<b>RNase</b>	<b>50.5</b>	<b>35.6</b>	<b>24</b>
<b>20% glycerol</b>	<b>DNase</b>	<b>107</b>	<b>111</b>	<b>52</b>
	<b>RNase</b>	<b>111</b>	<b>111</b>	<b>79</b>
<b>0.01M EtSH</b>	<b>DNase</b>	<b>49.5</b>	<b>50</b>	<b>65</b>
	<b>RNase</b>	<b>112</b>	<b>115</b>	<b>85</b>



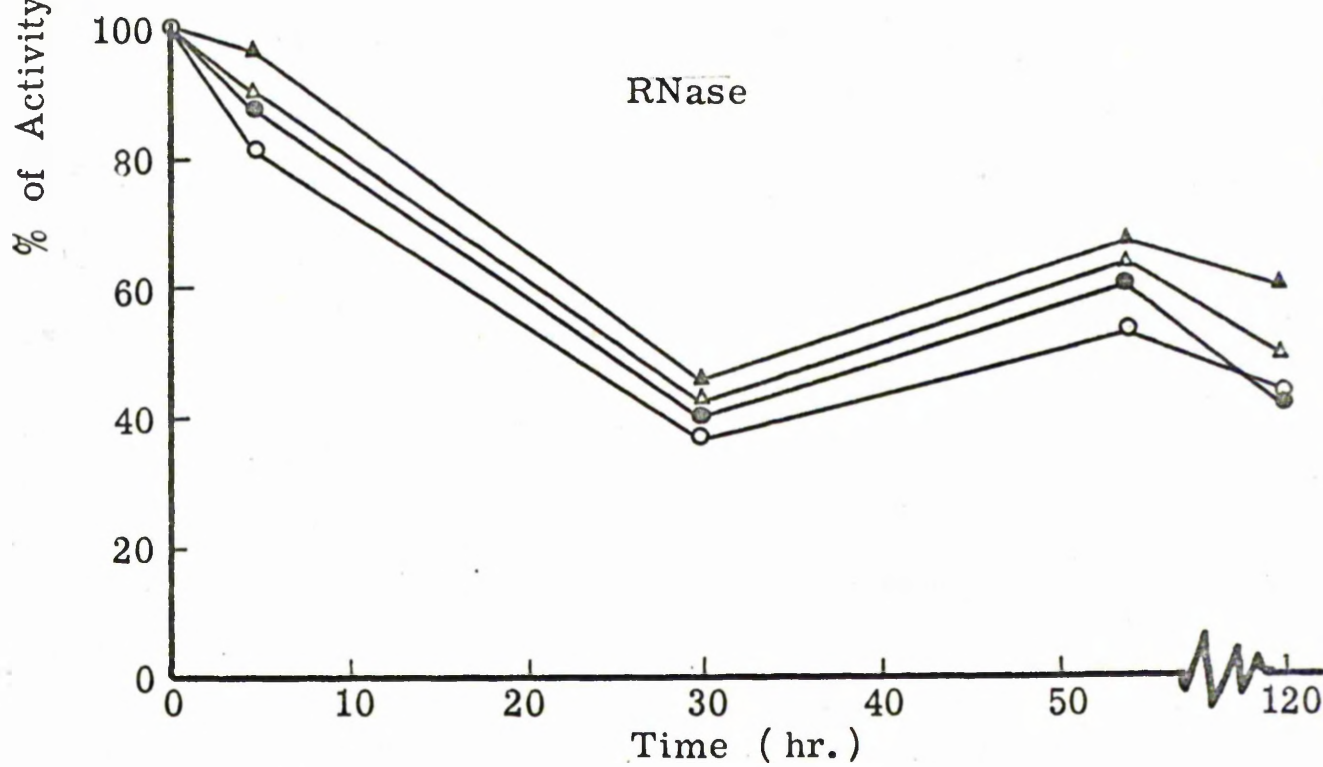
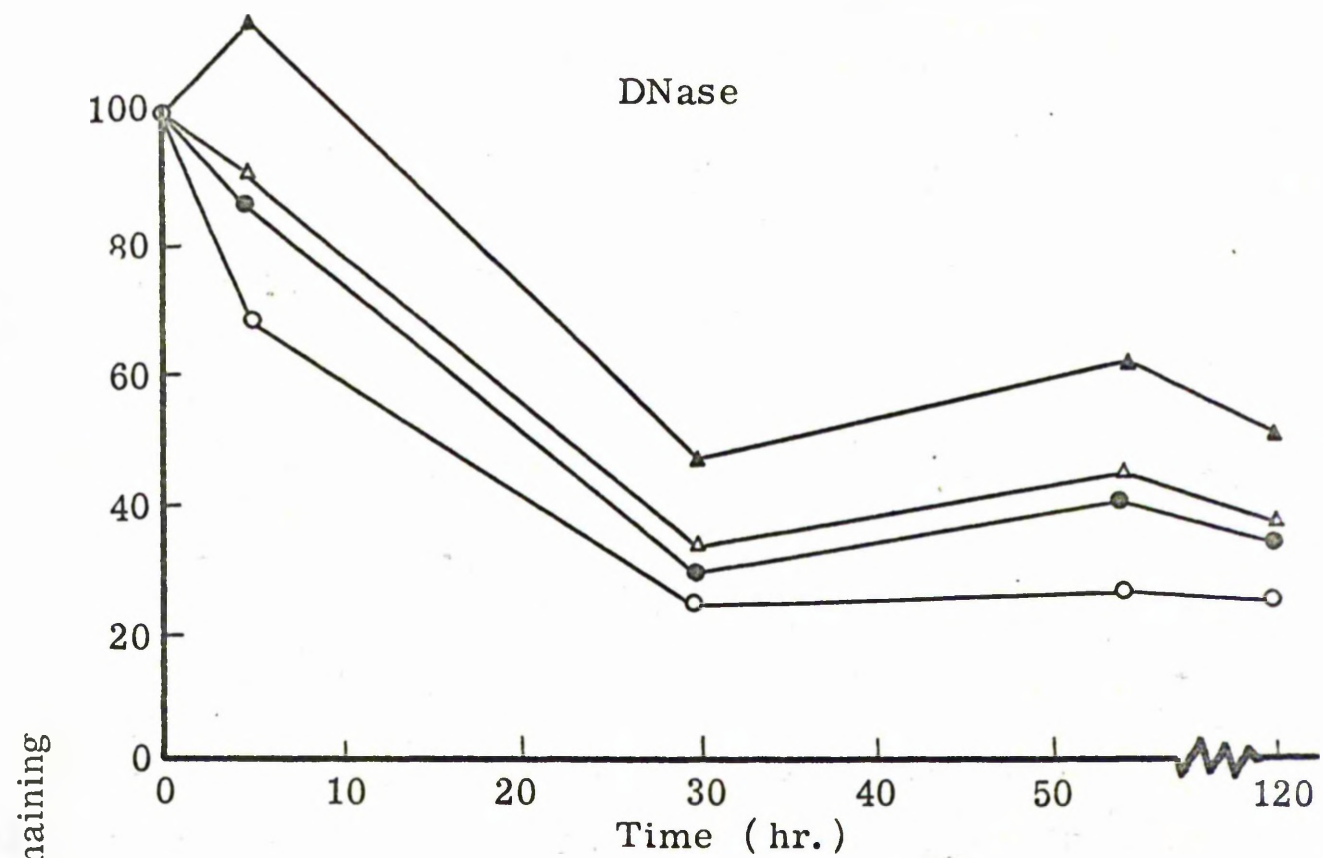
Figure 6.

The effect of 2-mercaptoethanol on the activity of the DEAE-cellulose effluent (fraction IV).

The DEAE-cellulose effluent was diluted with 0.01M tris-HCl buffer pH 8.0 to give 67  $\mu$ g. protein/ml. for DNase assays and 27  $\mu$ g. protein/ml. for RNase assays. The diluted solutions were placed in ice and assayed at various time intervals. The assay conditions were as described in this chapter section 2.4.1 and 3 except 5  $\mu$ moles 2-mercaptoethanol were replaced by (i) water —○— (ii) 0.5  $\mu$ moles 2-mercaptoethanol —●— (iii) 5  $\mu$ moles 2-mercaptoethanol —△— (iv) 50  $\mu$ moles 2-mercaptoethanol —▲—. The results are expressed as percentages of the zero time activities.



Figure 6.





### 3.4. Determination of the molecular weight of the nuclease

Gel filtration is a technique where molecules are separated according to molecular size, and for a homogeneous series of macromolecules, size and molecular weight are closely related. Andrews (1962) obtained evidence that this statement applies to a number of proteins using agar-gel columns as molecular sieves. With Sephadex G-75 and G-100 Andrews (1964) has shown that there is a linear relation between the elution volume and the  $\log_{10}$  (molecular weight) of a protein, and such a relation may be used to estimate the molecular weight of proteins. Therefore experiments were performed to show that this relation could be applied to the column used for the separation of the nuclease. The most convenient value to determine is  $R_{SA}$  which is the migration rate of a protein through the gel relative to that of serum albumin. The elution volumes of bovine  $\gamma$ -globulin, bovine serum albumin and horse heart cytochrome C were measured, the proteins being detected by their extinction at 280 m $\mu$ . Table 10 gives the  $R_{SA}$  values together with those of Andrews (1964). There is good agreement between the two sets of results and it was felt justifiable to plot these three elution volumes against  $\log_{10}$  (molecular weight) (Fig.7). The elution volume of the nuclease had previously been determined and its molecular



TABLE 10.

The relation between the elution volume and the molecular weight of a protein.

The elution volumes were determined on Sephadex G-75 column,  $1.7 \times 10^6$  cm., equilibrated with 0.01M imidazole-HCl buffer pH 6.8 and 0.005M with respect to 2-mercaptoethanol. The sample volume applied to the column was 1 ml. and contained 6 mg. of bovine  $\gamma$ -globulin, bovine serum albumin and cytochrome c. The values for the molecular weights were taken from Andrews (1964). The  $R_{SA}$  values were calculated from the elution volumes where  $R_{SA}$  is the migration rate of a protein through the gel relative to that of bovine serum albumin.



TABLE 10

Protein	Molecular Weight	Elution Vol. ml.	R <sub>SA</sub>	R <sub>SA</sub> <sup>*</sup>
γ-Globulin	160,000	72	1.12	1.09
Serum albumin	67,000	81	1.0	1.0
Cytochrome c	12,400	147	0.55	0.54
Rat liver nuclease		99	0.82	-

\*R<sub>SA</sub> values taken from Andrews (1964).



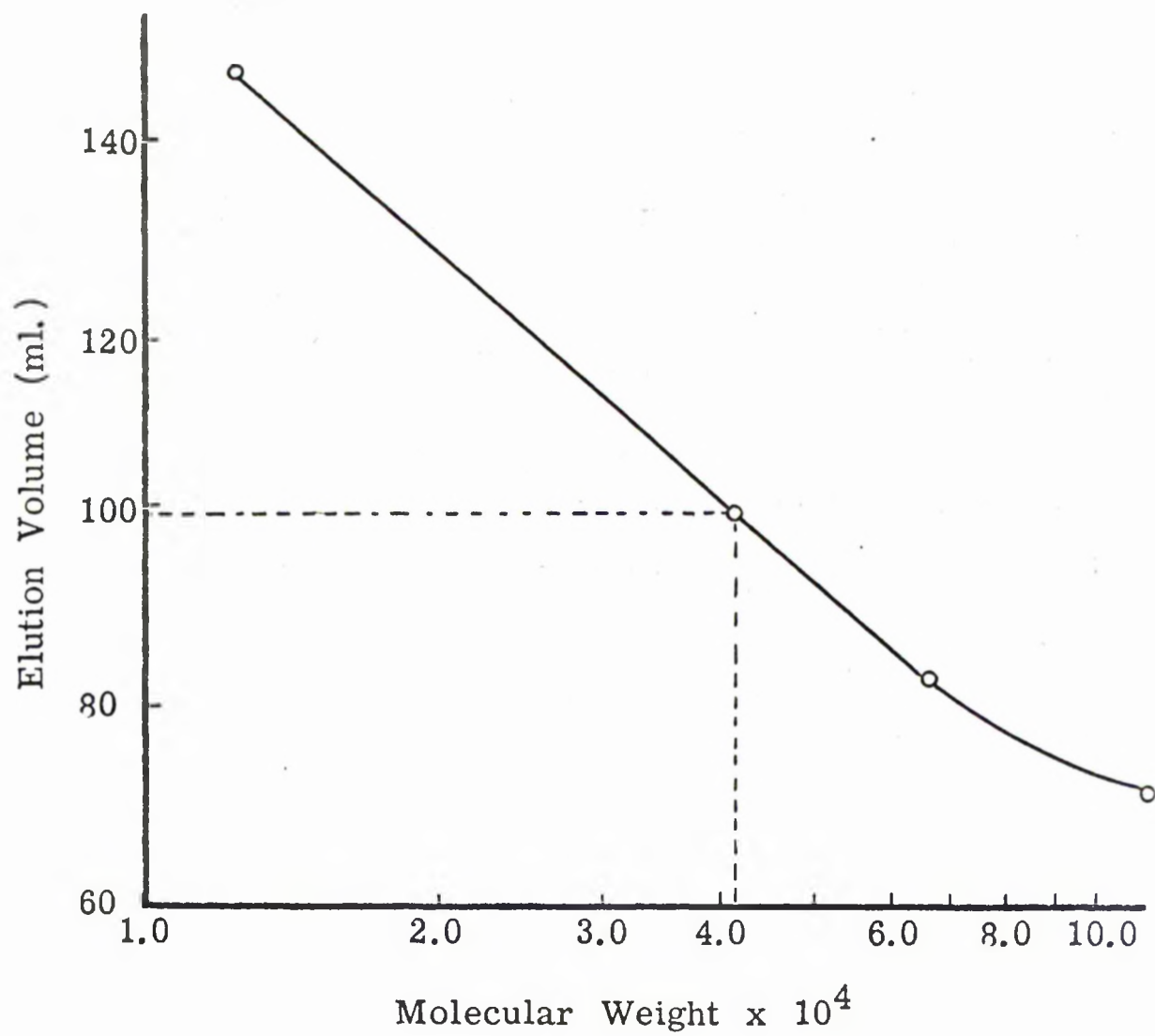
Figure 7.

The relation between the elution volume and the  $\log_{10}$ (molecular weight) of a protein.

The elution volumes of cytochrome c, bovine serum albumin, bovine  $\gamma$ -globulin and the purified liver nuclease were measured on a Sephadex G-75 column. The molecular weights were taken from Andrews (1964).



Figure 7.





weight was read directly from Fig. 7. A value of 44,000 was obtained though it must be realised that such a value is only approximate.

### 3.5. Properties of the liver nuclease

Before describing the properties of the nuclease, it is first necessary to consider the method of assay used. The method depending on the formation of acid-soluble products from an acid-insoluble substrate was chosen partly for its convenience and partly because it is possible to use as a substrate denatured DNA. The other two well documented methods depending on 1) the drop in viscosity 2) the change in hypochromicity require as a substrate native DNA.

The acid precipitability of oligonucleotides is largely dependent on the chain length, the limit being of the order of 6-12 units. However the acid precipitability is affected by the presence of proteins (Kurnick, 1962). The second point of importance is that the rate of production of acid-soluble oligonucleotides will presumably vary with the initial size of the substrate and with time. Thus smaller substrate molecules will contain more sites where hydrolysis will produce acid-soluble oligonucleotides. Their rate of production must vary with time. Initially the rate will be lower than the rate at which bonds are hydrolysed since the



majority of the products will still be large and acid-insoluble. But as these products are further degraded the number of sites where hydrolysis will produce acid-soluble products will increase. The acid-soluble products however can also act as substrates, but their hydrolysis will not be detected by this method, so that as the concentration of acid-soluble products increase, so the rate, as determined by this method, will decrease. Typically then a sigmoid curve would be expected, relating the rate with time, but the extent of the deviation from a straight line may not be great. In addition it must be emphasised that in the present work the use of duplicate assays has indicated an error of the order of 5%. These facts may mean that the sigmoid character may not be demonstrated. These considerations must be recognised in interpreting the following data.

The evidence obtained from the purification procedure indicated that the nuclease may have the ability to hydrolyse both DNA and RNA. Therefore the properties of DNase and RNase activities towards both substrates have been examined. In general, because of the higher rate of hydrolysis of RNA, less protein has been used in these assays than in the assays of DNase.



The time course of the hydrolysis of DNA and RNA was studied (Fig. 8). The rate was linear in the case of DNA for approximately 1 hr. in which time 30% of the substrate had become acid-soluble. With RNA linearity was found until 40% of the substrate had become acid-soluble. Beyond these values the rates declined until 100% solubilisation of the substrates occurred. Therefore it might be assumed that during the incubation time normally used, that is 30 min. the reaction rate is constant as only 15% of the substrate is converted to acid-soluble products. With RNA as a substrate there was direct proportionality between the rate of hydrolysis and the concentration of the enzyme over the range 0-10  $\mu\text{g. protein/ml.}$  (Fig.9). The rate of hydrolysis of DNA however showed a pronounced lag at the lower concentrations of protein, that is 0-10  $\mu\text{g. protein/ml.}$  Above this concentration there was direct proportionality. The lag was presumably related to the nature of the substrate and the assay method rather than a property of the DNase.

The study of the effect of the substrate concentration presented some difficulties. Preliminary experiments showed that the enzyme in both cases was rapidly saturated with respect to the substrate at a level where the rate of hydrolysis was constant for approximately 5-10 min. only. It was therefore necessary to estimate the rate over a



Figure 8.

The time course of the hydrolysis of DNA and RNA by the purified liver nuclease.

The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 0.25  $\mu$ moles  $\text{MgCl}_2$ , 5  $\mu$ moles 2-mercaptoethanol and 6.7  $\mu$ g. protein for DNase assays and 2.7  $\mu$ g. protein from fraction IV for RNase assays in a total volume of 0.5 ml. Incubation was at  $37^\circ$  for the indicated time intervals. The results are expressed as percentages of the amount of the substrate converted to acid-soluble products.

DNase      —○—  
RNase      —△—



Figure 8.

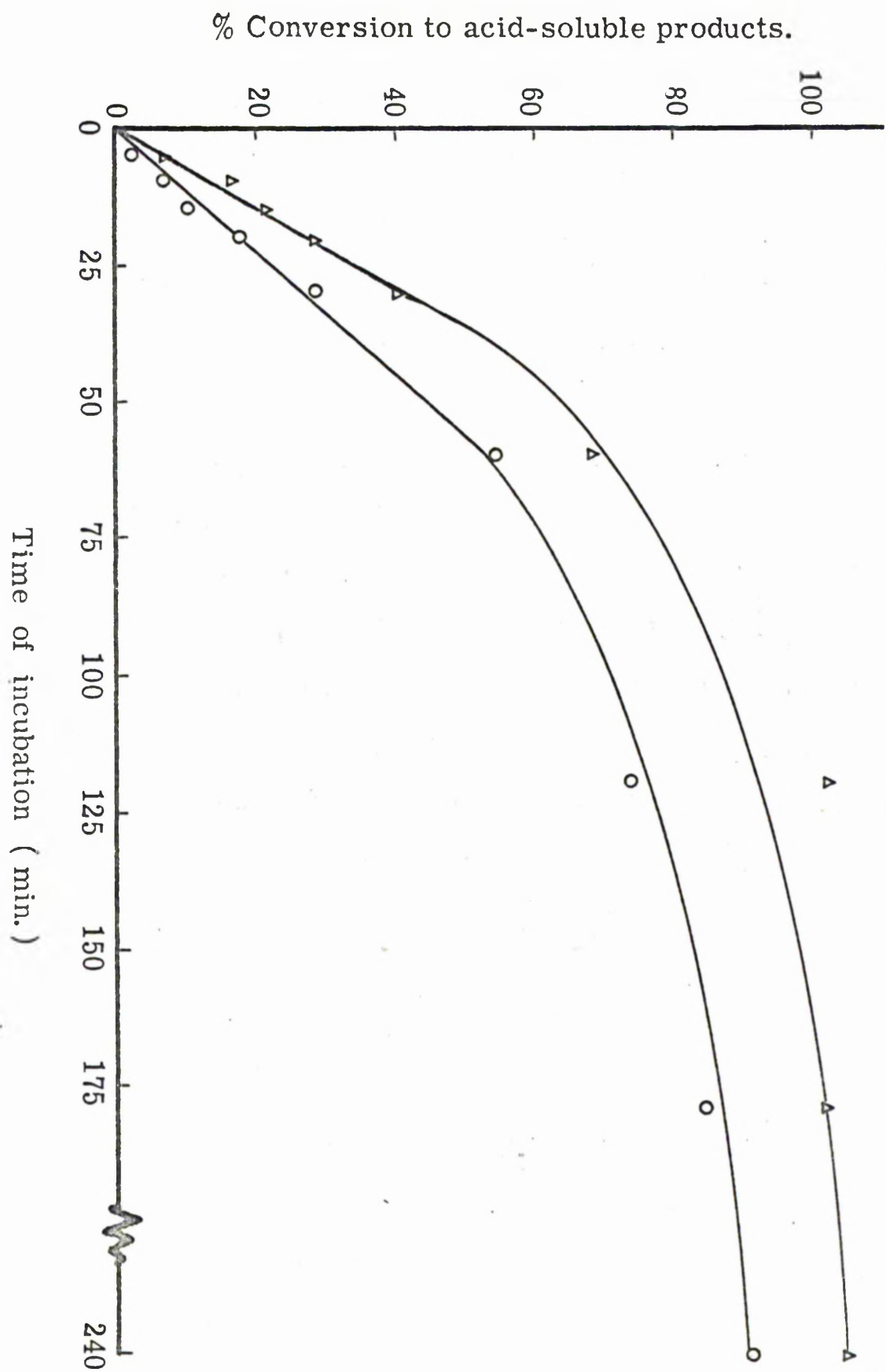




Figure 9.

The effect of the protein concentration on the rate of hydrolysis of DNA and RNA.

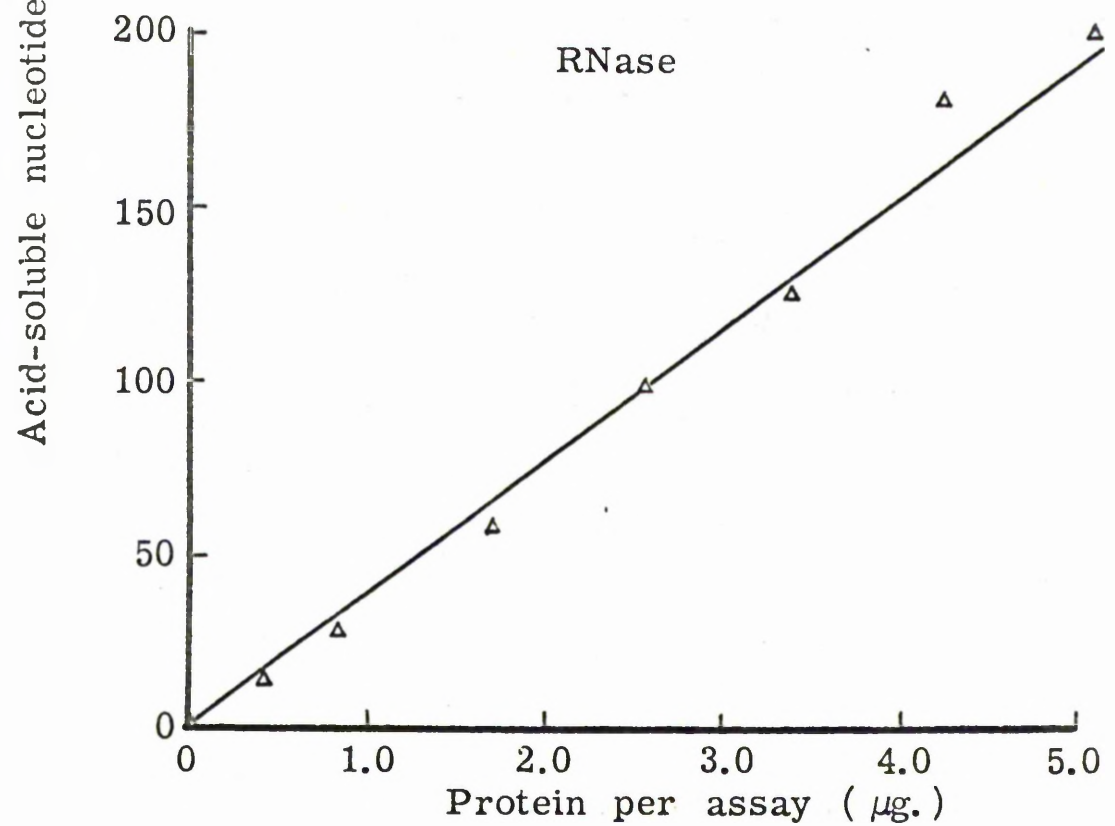
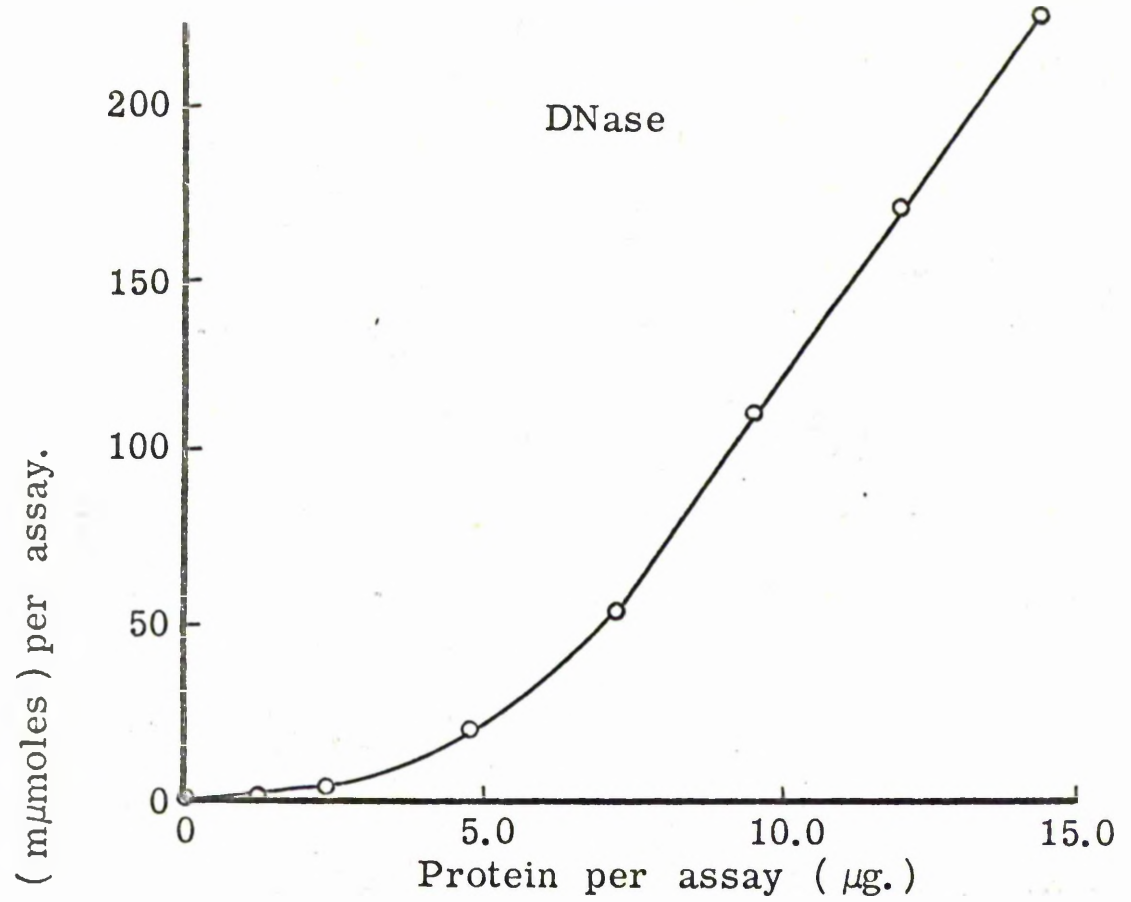
The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 0.25  $\mu$ moles  $MgCl_2$ , 5  $\mu$ moles 2-mercaptoethanol and various amounts of protein of fraction IV in a total volume of 0.5 ml. The assays were incubated at  $37^\circ$  for 30 min.

DNase —○—

RMase —△—



Figure 9.





correspondingly short period of time. To achieve this, procedure (2) (see this chapter section 2.4.1.) for assaying DNase and RNase was used. After preincubating the assay medium for 2 min. 0.5 ml. samples were removed at 0, 2, 4, 6, and 8 min. after the addition of the enzyme. Also the concentration of the substrate was related to the phosphorus content since this would provide a more accurate estimation of the substrate concentration than is normally required. The results suggest that the hydrolysis of DNA and RNA follow Michaelis-Menten kinetics (Fig. 10). The  $K_m$  for the two activities are approximately the same, of the order of  $8 \times 10^{-5} M$ .

The effect of pH was studied in a number of different buffer systems. It was found that in comparison to those used in Fig. 11, that is imidazole-HCl and tris-HCl, phosphate and glycine-NaOH buffers gave comparatively low activities. The effect of glycine may be related to its ability to chelate metal ions. The two activities showed a sharp pH optimum about 6.8 - 7.0. On the acid side this was rapidly reduced, so that at pH 5.6 only 5% of both activities remained. On the alkaline side, the fall in activity was less pronounced and at pH 8.8 about 35% remained.

The two activities showed an absolute requirement for  $Mg^{2+}$  and  $Mn^{2+}$  ions (Fig. 12). The requirement is approximately



Figure 10.

The effect of the substrate concentration on the rate of hydrolysis of DNA and RNA by the purified liver nuclease.

The method of assay used was that described in this chapter section 2.4.1 procedure (2). For DNA a concentration of 21  $\mu\text{g}$ . protein/ml. was used and for RNA 5  $\mu\text{g}$ . protein/ml., the protein being from fraction IV. The rates were determined by removing samples at 0, 2, 4, 6 and 8 min. intervals and the initial rate was estimated from these values. The rate is expressed as  $\mu\text{moles}$  acid-soluble nucleotides released in 10 min.

DNA and RNA concentrations are expressed in terms of  $\mu\text{moles}$  of phosphorus.

DNase      —○—  
RNase      —△—



Figure 10.

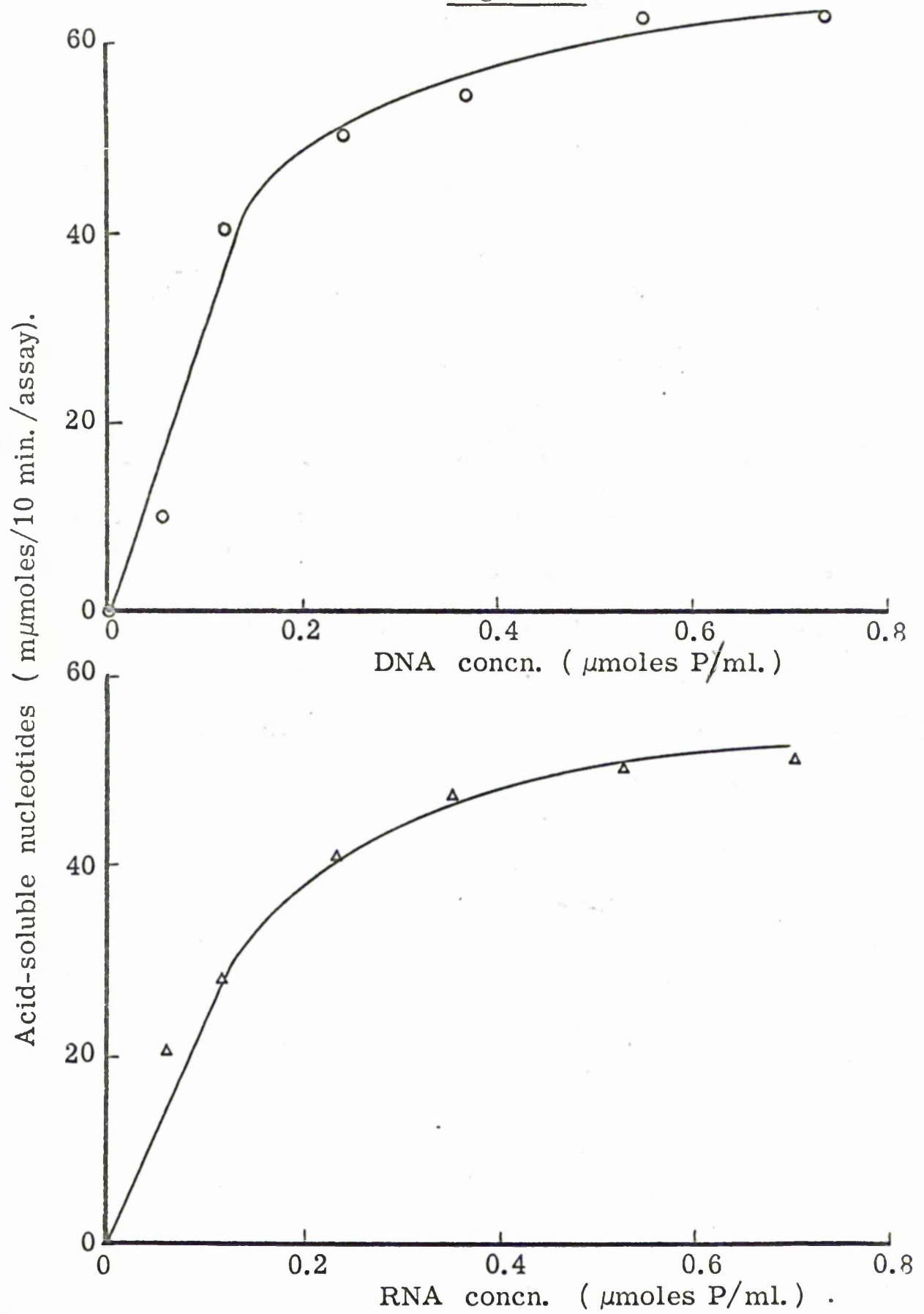




Figure 11.

The effect of pH on the activity of the purified liver nuclease.

The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 0.25  $\mu$ moles  $\text{MgCl}_2$ , 5  $\mu$ moles 2-mercaptoethanol and 25  $\mu$ moles buffer in a total volume of 0.5 ml. DNase assays contained 1.1  $\mu$ g. and RNase assays 0.6  $\mu$ g. protein of fraction V. The buffers used were (i) acetate at pH 5.1 and 5.6 (ii) imidazole-HCl at pH 6.0-7.6 (iii) tris-HCl at pH 7.5-8.8. The incubation was at 37° for 30 min.

DNase    —○—  
RNase    —△—



Figure 11.

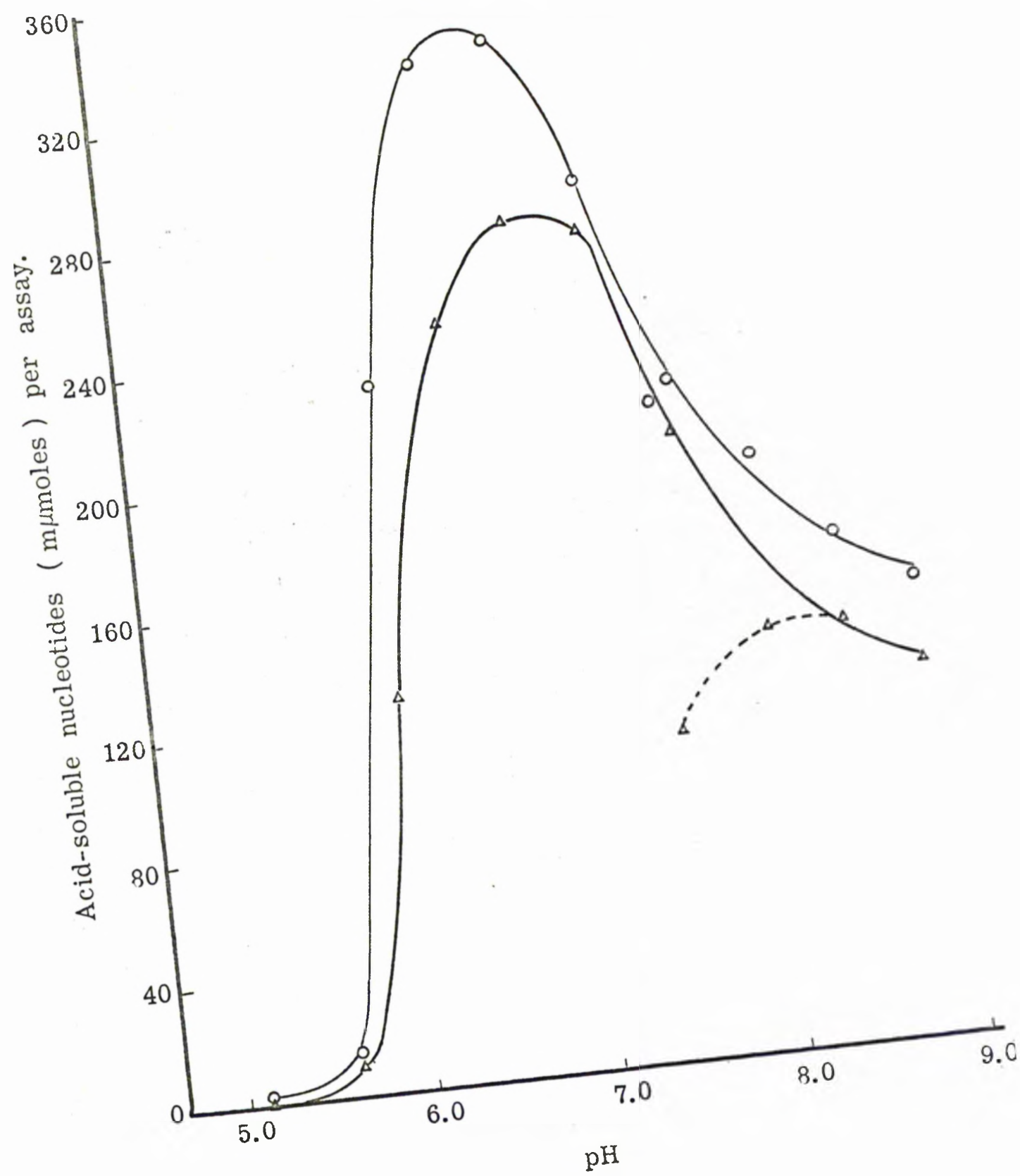




Figure 12.

The effect of  $Mg^{2+}$  and  $Mn^{2+}$  ions on the activity of the purified liver nuclease.

The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 5  $\mu$ moles 2-mercaptoethanol and 0.9  $\mu$ g. for DNase assays and 0.45  $\mu$ g. protein of fraction V for RNase assays in a total volume of 0.5 ml.  $MgCl_2$  and  $MnCl_2$  were added to give the indicated molarities. The assays were incubated at  $37^\circ$  for 30 min.

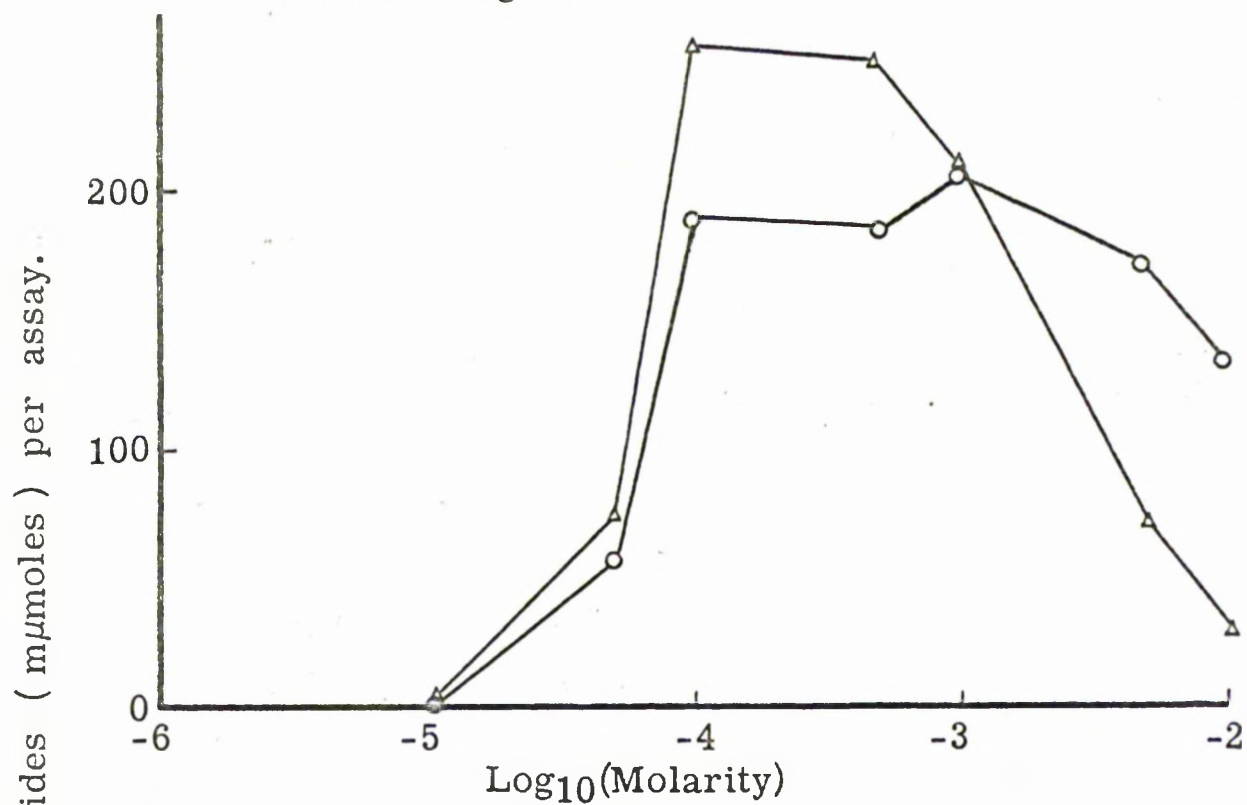
DNase —○—

RNase —△—

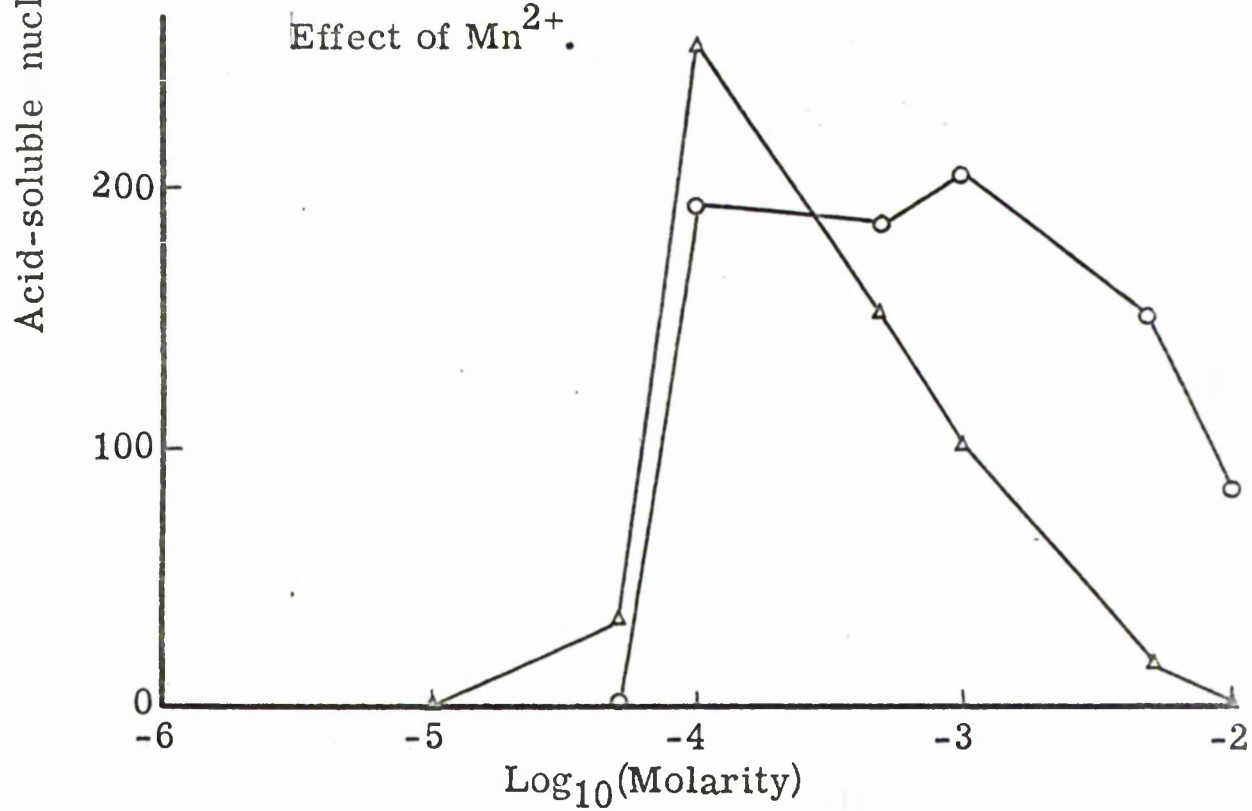


Figure 12.

Effect of  $\text{Mg}^{2+}$ .



Effect of  $\text{Mn}^{2+}$ .





the same for both cations and for both activities, that is the optimum concentration for DNase about  $10^{-4}M$   $Mg^{2+}$  and  $Mn^{2+}$ , as also was the case for RNase. The plots of activity against concentration of  $Mg^{2+}$  and  $Mn^{2+}$  were very similar. However these plots for DNase differed from RNase in that the optimal activity remained in the case of DNase over the range  $10^{-4}$  -  $10^{-3}M$   $Mg^{2+}$  or  $Mn^{2+}$ , whereas beyond  $10^{-4}M$  there was marked inhibition of the RNase by  $Mn^{2+}$  and less marked inhibition by  $Mg^{2+}$ .

In the absence of  $Mg^{2+}$ ,  $Ca^{2+}$  showed no stimulatory effect (Fig.13). In the presence of the optimal concentration of  $Mg^{2+}$ ,  $Ca^{2+}$  showed increasing inhibition with increasing concentration, 50% inhibition of the DNase and RNase being evident at 3.5 mM and 1mM respectively. Monovalent cations inhibited both activities markedly, though over a higher concentration range. Thus the DNase and RNase were inhibited 50% at 50 mM and 10mM respectively (Fig.14).

The effect of heat was examined at two pHs, 5.1 and 7.2 (Fig.15). The enzyme solution used was that obtained from Sephadex G-75 and had previously been made 20% with respect to glycerol and 0.04M with respect to 2-mercaptoethanol. This solution was diluted 6 times, so that heating of the enzyme occurred in 3% glycerol and 6 mM 2-mercaptoethanol.



Figure 13.

The effect of  $\text{CaCl}_2$  in the absence and in the presence of the optimum concentration of  $\text{MgCl}_2$  on the activity of the purified liver nuclease.

The assay medium contained 300  $\mu\text{g}$ . denatured DNA or RNA, 25  $\mu\text{moles}$  0.01M imidazole-HCl buffer pH 6.8, 5  $\mu\text{moles}$  2-mercaptoethanol and 0.9  $\mu\text{g}$ . for DNase assays and 0.45  $\mu\text{g}$ . protein of fraction V for RNase assays in a total volume of 0.5 ml. For assays in the presence of  $\text{MgCl}_2$  0.25  $\mu\text{moles}$  were added. The assays were incubated at  $37^\circ$  for 30 min.

DNase —○—

RNase —△—



Figure 13.

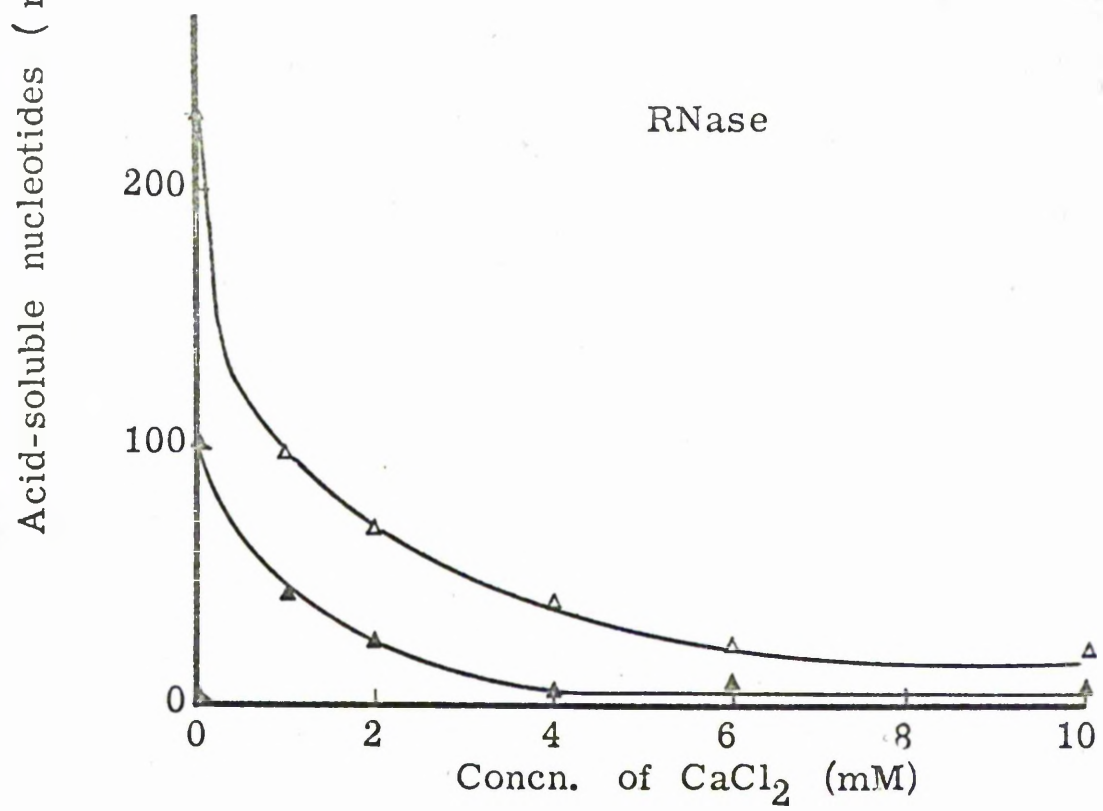
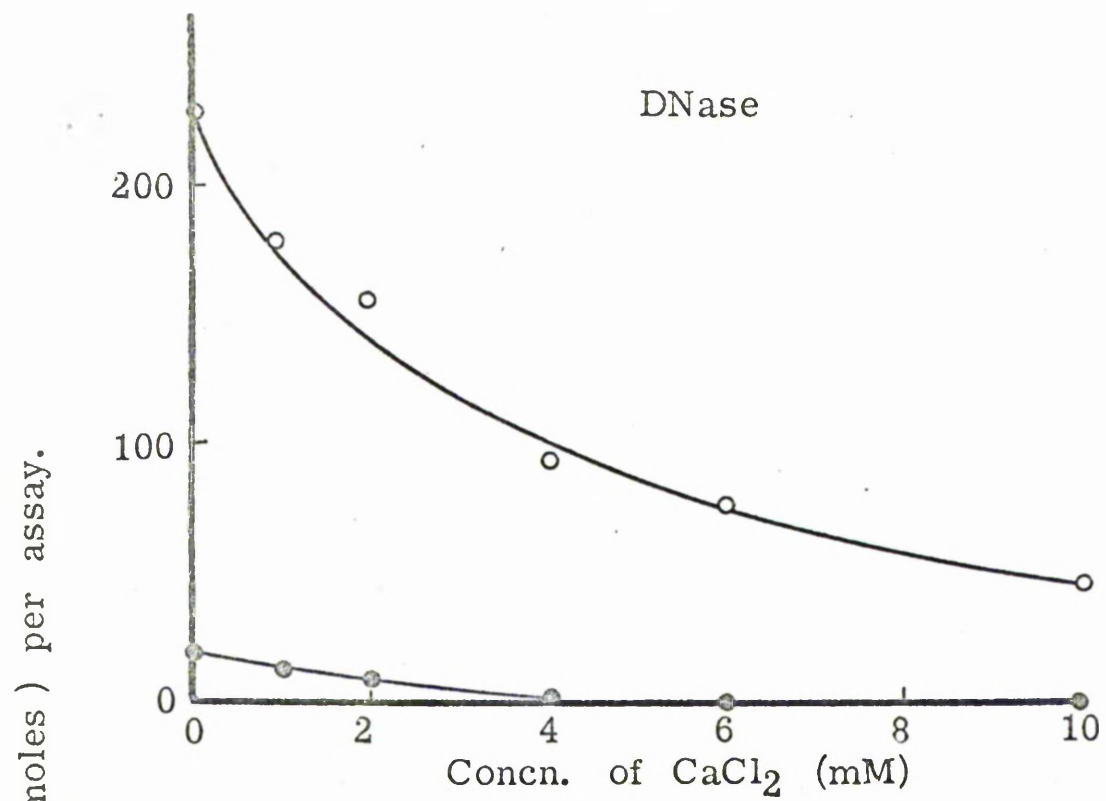




Figure 14.

The effect of NaCl on the activity of the purified liver nuclease.

The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 0.25  $\mu$ moles  $MgCl_2$ , 5  $\mu$ moles 2-mercaptoethanol and 3  $\mu$ g. for DNase assays and 1.5  $\mu$ g. protein of fraction V for RNase assays in a total volume of 0.5 ml. NaCl was added to give the indicated molarities. The assays were incubated at 37° for 30 min.

DNase —○—

RNase —△—



Figure 14.

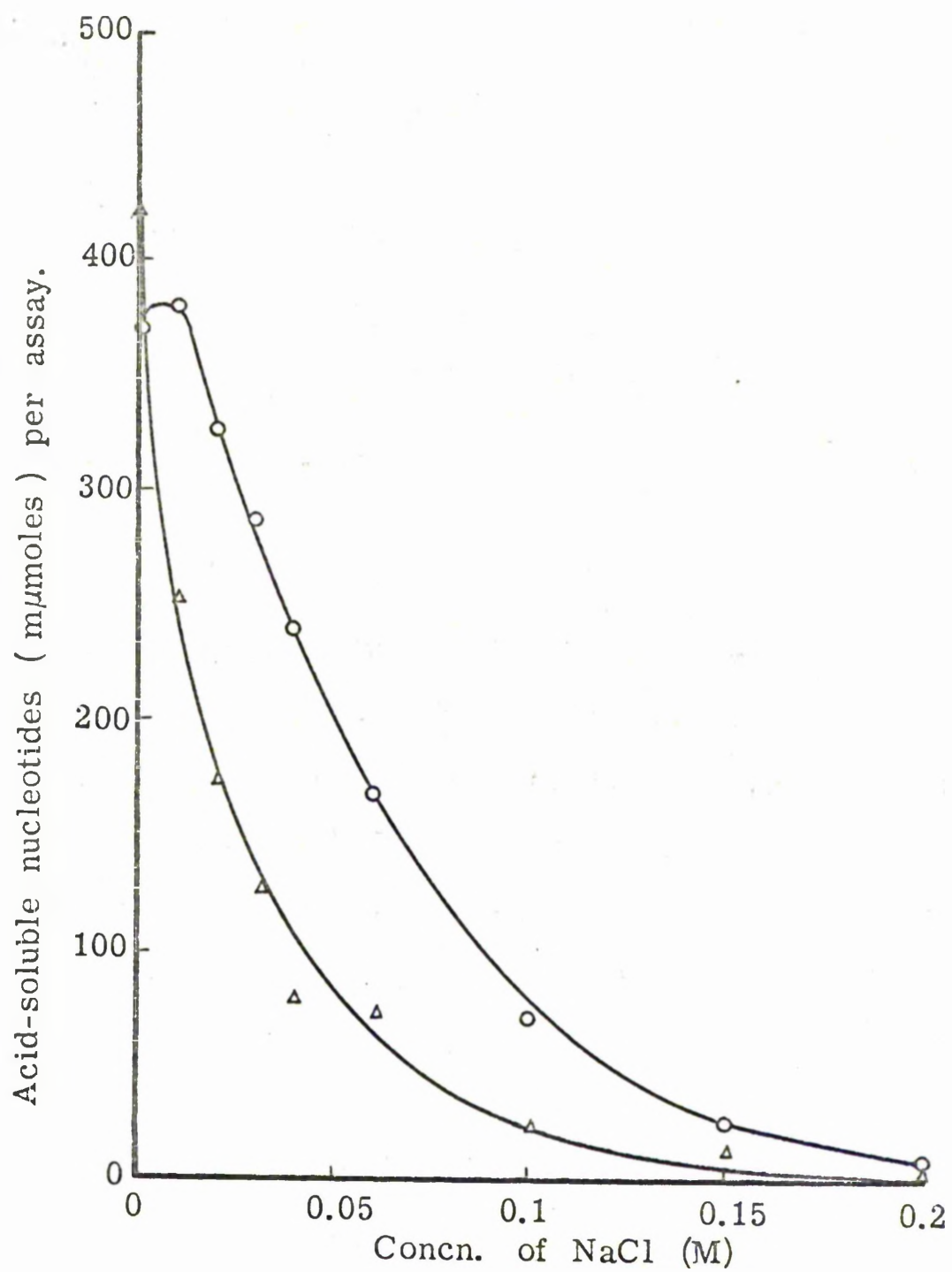




Figure 15.

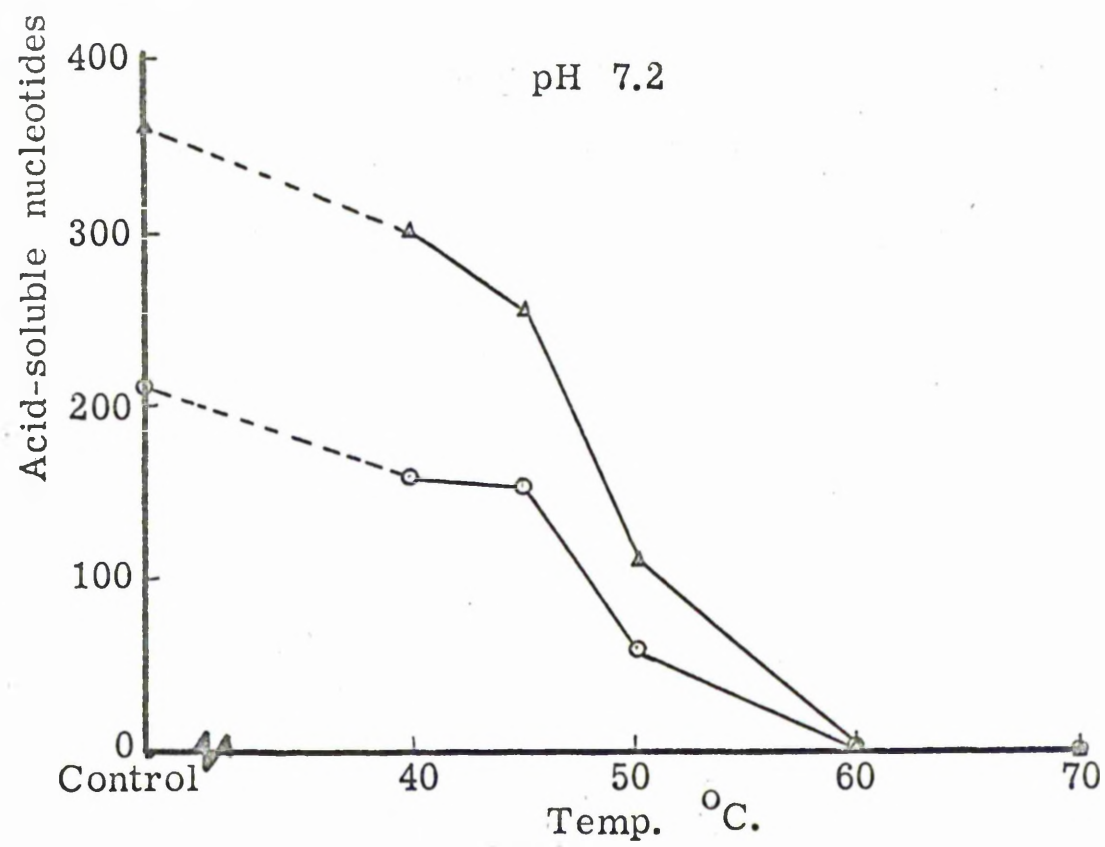
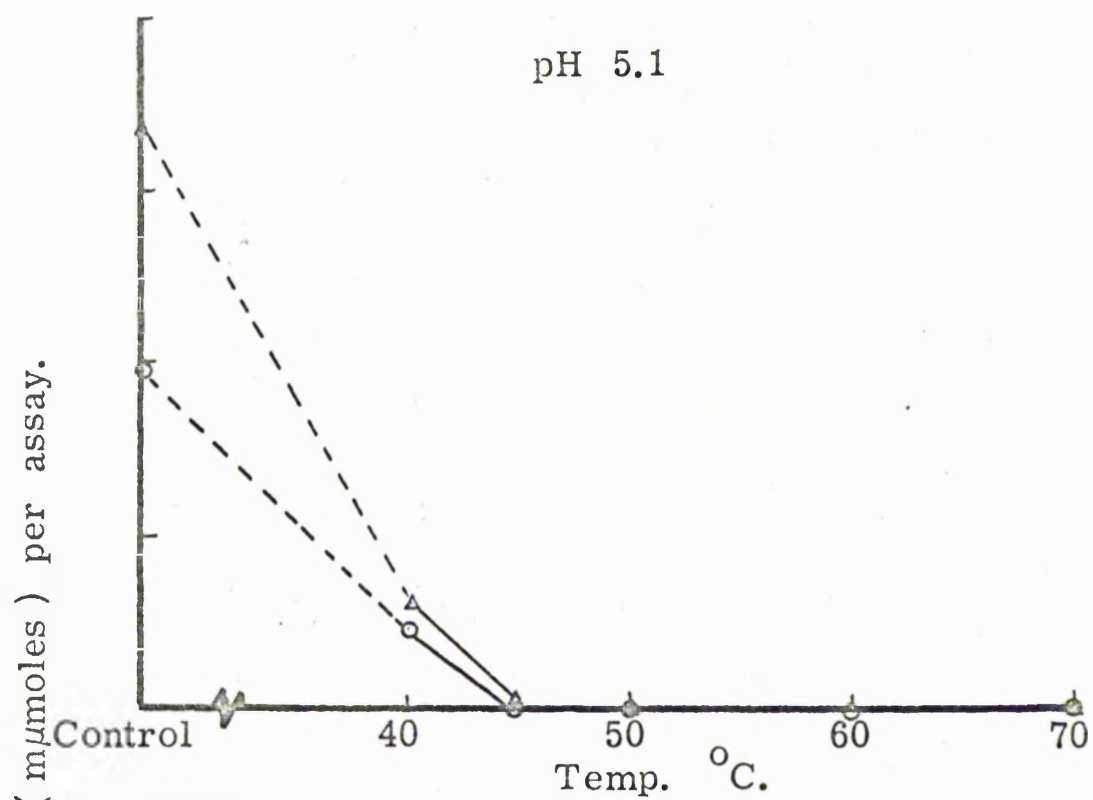
The effect of heating on the activity of the purified liver nuclease.

Samples containing 11  $\mu$ g. protein of fraction V and 50  $\mu$ moles acetate buffer pH 5.1 or imidazole-HCl buffer pH 7.2 in a volume of 1.2 ml. were heated at 40°, 45°, 50°, 60° and 70° for 5 min. and then immediately cooled in ice until assayed. The control was kept in ice until assayed. The assay medium contained 300  $\mu$ g. denatured DNA or RNA, 25  $\mu$ moles imidazole-HCl buffer pH 6.8, 0.25  $\mu$ moles  $MgCl_2$ , 5  $\mu$ moles 2-mercaptoethanol and 0.1 ml. of the samples in a total volume of 0.5 ml. The assays were incubated at 37° for 30 min.

DNase —○—  
RNase —△—



Figure 15.





These constituents may have influenced the results obtained. It was found that at pH 5.1 both activities were rapidly inactivated, thus only 2% remained after heating at 45° for 5 min., whereas at pH 7.2 50% of both activities remained after heating at 47° for 5 min.



#### 4. Discussion

The purification procedure is satisfactory in that the contaminating activities, phosphomonoesterase, phosphodiesterase I and II and DNase II are reduced to a very low level and this means that the specificity of the DNase may be studied. The overall purification for the procedure is 700 fold, and though it has been found that each individual stage varies in the increase in the specific activity that it may give, the overall result appears to be the same. The procedure is relatively simple, and it is possible to accomplish the purification in 2 days though 3 days are normally taken. The low yield however is a distinct disadvantage of the method, and it is here where an improvement would be desirable. The low yield of units may be related to the observed instability of the DNase. There are two points that are important in this respect. The effect of 2-mercaptoethanol and the thiol reagents, sodium p-hydroxymercuribenzoate and iodoacetamide, indicate that the DNase does not have a thiol group within the active site. Also the magnitude of the effects observed would indicate that if thiol groups are the sites of the partial effects of 2-mercaptoethanol and the two thiol reagents then these groups are probably not directly involved in the binding of the substrates. The thiol groups are then likely to act as partially determining the secondary and tertiary structure of the DNase. The effect of glycerol on the stability of the DNase may be



related to the possibility that the enzyme is membrane bound. Further support for this contention is provided by the sudden change in the stability of the enzyme after passage through DEAE-cellulose. Such an observation suggests that after acetone fractionation the DNase exists still as a complex possibly involving other proteins. The DNase would then be stabilised in this complex. Passage through DEAE-cellulose might remove such proteins from the complex where they were stabilising the DNase. Such suggestions however raise the question of whether the observed activities of the enzyme are the actual activities of the native protein.

The activities towards denatured DNA, native DNA and RNA appear to be intrinsic properties of the same protein. Support for this view comes from the observation that the ratio of the three activities is relatively constant throughout the purification procedure, and in particular by the result that on Sephadex G-200 and G-75 the three activities are exactly superimposed. Further support comes from the result that the ratio of the rates of hydrolysis of single stranded DNA to native DNA is very similar to the ratio of the rates of hydrolysis of single stranded RNA to double stranded (sRNA). Finally the properties of the DNase and RNase with respect to pH,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$  and Na requirements are similar and the heat stability of the two activities at two different pHs are



superimposable. The evidence presented therefore supported the suggestion that one protein is responsible for the three activities or alternatively there are two or three proteins, with one activity each, that are very similar in size and requirements, for activity.

However the results on the storage of the DNase and RNase would seem to indicate that two distinct proteins are involved, for the two activities have differing stabilities under the various conditions, RNase being more stable than DNase. Nevertheless one might argue that only one protein is involved, the DNase activity of the protein requiring a more specific configuration of the protein molecule than the RNase activity.



### CHAPTER III

#### The Specificity of the Liver Nuclease



## 1. Introduction.

The value of determining the specificity of a nuclease is two fold. Firstly it is necessary for the characterisation of the enzyme so that it may be distinguished from other similar enzymes. This is particularly evident for the RNase activity for there are three distinct RNases in the liver; acid RNase that is located in the lysosomes (Roth, 1957; Mavor & Greco, 1962), alkaline RNase located in the nuclei, termed RNase I (Heppel, Ortiz & Ochoa, 1956), and an alkaline RNase located in the mitochondria, termed RNase II (Roth, 1957; Beard & Razzell, 1964). Secondly, nucleases of known specificity are useful in the study of the structure and base sequences of nucleic acids. The more specific a nuclease is towards its substrate, the more value it is. However few nucleases exhibit absolute specificity apart from RNase T<sub>1</sub>, which is specific for the linkage -GpXp-, and pancreatic RNase, which is specific for the linkage -PypXp-. Absolute specificity for denatured DNA is shown by E. coli exonuclease I and by lamb brain endonuclease, and such a specificity has many applications.

There are three aspects of specificity of the purified nuclease described in this thesis that remain to be answered. The first aspect, that of specificity for the substrate, has not been answered completely, though the nuclease would



appear to lack specificity towards DNA and RNA. The remaining three points are (i) whether the attack is endonucleolytic or exonucleolytic (ii) whether the oligonucleotides are terminated by 3'- or 5'-phosphate groups (iii) whether the enzyme shows any preference for the bases on the 3' and or 5' side of the hydrolysed bond. These three aspects of the specificity were examined for both the DNase and RNase, and from such a comparison further evidence might be gained to determine whether one or two proteins are involved.



## 2. Experimental.

The techniques that are common to chapters 2 and 3 will not be described again in this section, except in so far as they have been modified.

### 2.1. Materials.

Salmon sperm DNA was purchased from the California Corporation for Biochemical Research, highly polymerised yeast RNA from the British Drug Houses, crude snake venom (Crotaleus adamanteus) from the Sigma Chemical Company. Adenosine, guanosine, cytidine, and adenosine 2'- and 3'-monophosphate were purchased from the California Corporation for Biochemical Research, inosine from the Nutritional Biochemicals Corporation, Cleveland, Ohio, deoxyinosine and thymidine from the Sigma Chemical Company, deoxyadenosine, deoxyguanosine, deoxycytidine and adenosine 5'-monophosphate from Schwarz Bioresearch, Inc., Mount Vernon, New York.

### 2.2. Enzymes.

Pancreatic DNase I (stock DN-C), pancreatic RNase and spleen phosphodiesterase were purchased from the Sigma Chemical Company, E. coli alkaline phosphatase from the Nutritional Biochemicals Corporation.

#### Purification of snake venom phosphodiesterase.

As the procedure for the purification of snake venom phosphodiesterase was taken from several papers, the source



of the procedure used will be indicated at each step.

Step 1. Acetone fractionation. (Rjork, 1963)

2 g. crude venom of Crotaleus adamanteus was dissolved in 120 ml. cold water, and was stirred for 30 min. in an ice bath. The solution was filtered on a Buchner funnel through Whatman No. 3mm filter paper. The clear solution was mixed with 80 ml. ice cold 0.5M acetate buffer pH 3.8 and 145 ml. acetone previously cooled to  $-15^{\circ}$  were added rapidly to attain 42% acetone concentration (volumes were assumed to be additive). After stirring in an ice bath for 30 min. the precipitate was removed by centrifugation at 10,000 x g for 20 min. at  $0^{\circ}$ . The supernatant solution was transferred to a glycol bath at  $-15^{\circ}$  and 19 ml. acetone (cooled to  $-15^{\circ}$ ) were added with stirring. The acetone concentration was 45%. After stirring for 2 hr. the precipitate was removed by centrifugation at 600 x g for 20 min. at  $-15^{\circ}$  and discarded. The supernatant liquid was replaced in the glycol bath at  $-15^{\circ}$  and a further 36 ml. acetone (cooled to  $-15^{\circ}$ ) were added, giving a concentration of 50%. The solution was stirred for 1 hr. and then was centrifuged at 600 x g for 20 min. at  $-15^{\circ}$ . The precipitate was dissolved in 20 ml. ice cold water.

Step 2. Ethanol fractionation. (Williams et al., 1961)

Ice cold water and 1M acetate buffer pH 6.0 were added



to the solution to give an extinction at 280 mμ of 10 and a final molarity of the acetate buffer of 0.1M. 0.5 vol. ethanol at 0° were added, giving 33% ethanol concentration, and the solution was centrifuged at 600 x g for 20 min. at 0°. The supernatant liquid was made 66% with respect to ethanol by the addition of 1.5 vol. ethanol, cooled to -15°, and the solution was centrifuged at 600 x g for 20 min. at -15°. The precipitate was dissolved in 0.1M tris-HCl buffer pH 8.9 and the volume adjusted to give an extinction at 280 mμ of 10.

Step 3. Ethanol fractionation. (Williams et al., 1961)

This step was repeated. The precipitate obtained between 33-66% ethanol was dissolved in ice cold water.

Step 4. Acetone fractionation. (Sulkowski & Laskowski, 1962)

The volume was adjusted to give an extinction at 280 mμ of 11 with ice cold water and 0.1 vol. 2M acetate buffer pH 4.0 were added. Acetone, cooled to -15°, was added to the solution to give a concentration of 44%, and the solution was then transferred to a glycol bath at -15° for 30 min. The solution was centrifuged at 600 x g for 20 min. at -15°. The supernatant liquid was brought to 50% with acetone, and transferred to a glycol bath at -15° for 1 hr. The precipitate was collected by centrifugation at 600 x g for 20 min. at -15° and was then dissolved in a small volume of 0.05M acetate buffer pH 6.0 and dialysed overnight against



2 litres of the same buffer.

Step 5. CM-cellulose. (Felix et al., 1960)

The dialysed solution was placed on a column of CM-cellulose (1 x 5 cm.) previously equilibrated with 0.05M acetate buffer pH 6.0. The column was then washed with 20 ml. 0.05M acetate buffer pH 6.0 which eluted the first peak of protein but little phosphodiesterase. The buffer was changed to 0.2M acetate buffer pH 6.0 and 2 ml. fractions were collected. The fractions containing activity towards p-nitrophenyl-pT were pooled and freeze-dried.

Step 6. DEAE-cellulose. (Felix et al., 1960)

The freeze-dried material was dissolved in 1 ml. 0.01M tris-HCl buffer pH 8.9 and dialysed against 1 litre of the same buffer overnight at 0°. The dialysed solution was applied to a column of DEAE-cellulose (1 x 5 cm.) previously equilibrated with 0.01M tris-HCl buffer pH 8.9. The same buffer removed the majority of the phosphodiesterase. Those fractions containing activity were pooled, and then 0.5 ml. fractions were stored separately at -70°.

The snake venom phosphodiesterase was assayed as follows: 0.1 ml. of a diluted solution of the enzyme was incubated at 37° for 10 min. with 2 µmoles p-nitrophenyl-pT and 20 µmoles tris-HCl buffer pH 8.9 in a total volume of 0.5 ml. The reaction was stopped by the addition of 2.5 ml. 0.1M NaOH.



The extinction at 400 mμ was measured. The purified snake venom phosphodiesterase released 100 μmoles p-nitrophenol/min./ ml. of the undiluted solution.

The purchased spleen phosphodiesterase had a specific activity of approximately 23 μmoles nucleotide released/hr./ mg. of protein.

E. coli alkaline phosphatase was assayed as follows: 0.1 ml. diluted enzyme solution was incubated at 37° for 10 min. with 2 μmoles p-nitrophenyl phosphate and 20 μmoles tris-HCl buffer pH 8.0. The reaction was stopped with the addition of 2.5 ml. 0.1N NaOH. The extinction at 400 mμ was measured. The enzyme released 1.5 moles p-nitrophenol/min./ ml. of the undiluted enzyme.

Snake venom phosphodiesterase and spleen phosphodiesterase showed no detectable phosphatase under the following conditions: for snake venom phosphodiesterase, 0.1 ml. undiluted enzyme solution was incubated with 15 μmoles 5'-AMP, 50 μmoles tris-HCl buffer pH 8.9 in total volume of 0.5 ml. at 37° for 4 hr. 0.05 ml. of the solution was spotted on Whatman No. 3mm paper and the chromatogram was developed in a descending manner for 12 hr. using 95% ethanol-1M ammonium acetate pH 7.5 (70:35 v/v). The solvent separated AMP from adenosine. No adenosine however was detected. For spleen phosphodiesterase, 1 mg. of the enzyme protein was incubated with 15 μmoles 2'-



and 3'-AMP, 50 umoles sodium succinate buffer pH 6.5 in a total volume of 2 ml. at 37° for 6 hr. 0.05 ml. of the solution was chromatographed as above. Again no adenosine could be detected.

The Sephadex G-75 pooled fraction of the liver nuclease was used in all the experiments to be described.

### 2.3. Inactivation of E. coli alkaline phosphatase.

The procedure described by Beers (1961) of shaking with chloroform for 1 hr. at room temperature did not inactivate the enzyme under the conditions used for dephosphorylation of oligonucleotides. Since the complete inactivation of the phosphatase was critical to the experiments a more vigorous method was employed. It consisted of refluxing the solution with an equal volume of ethanol for 1 hr. The ethanol was then allowed to evaporate. An equal volume of chloroform was added and the mixture was shaken vigorously for 2 hr., after which the two layers were separated by centrifugation and the upper aqueous layer was removed.

### 2.4. Preparation of triethylammonium bicarbonate buffers.

1.0M triethylammonium bicarbonate buffer pH 8.6 was prepared by passing CO<sub>2</sub> through water containing the required volume of triethylamine until the pH reached 8.6. 0.01M and 0.05M buffers were prepared similarly.



## 2.5. Column chromatography of oligonucleotides.

DEAE-cellulose was prepared as described in chapter 2 section 2.6.3. The cellulose was equilibrated with 0.05M triethylammonium bicarbonate buffer pH 8.6. The column, 2 cm. diameter by 40 cm., was packed with pressure (15 cm. Hg.) and was washed with 0.01M triethylammonium bicarbonate buffer pH 8.6 until the pH of the effluent was the same as the eluent.

The digest was applied to the column at an ionic strength of 0.01 and pH 6.8. When the digest had entered the cellulose, the surface of the column was washed with 50 ml. 0.01M triethylammonium bicarbonate buffer pH 8.6 before a gradient was applied. The gradient consisted of 1.4 litres 0.01M triethylammonium bicarbonate buffer pH 8.6 and 1.4 litres 1.0M of the same buffer, the containing vessels being connected in an open system. A hydrostatic pressure of 1 m. above the top of the column was used. The flow rate was 4ml./ min. and 16 ml. fractions were collected.

The resulting gradient was measured by determining the nitrogen content of selected fractions. 1 ml. of a suitably diluted fraction was digested with 0.2 ml. concentrated  $H_2SO_4$ . The resultant ammonium sulphate was estimated after the solution had been neutralised with alkali, using Nessler's reagent. The molarity of the buffer was calculated from this value.



The fractions were pooled according to the peaks of ultraviolet absorption and the pooled fractions were freeze-dried. It was found that after approximately 48 hr. the buffer had been completely removed as judged by the absence of its characteristic smell. The remaining material was dissolved in a small volume of water and stored at  $-20^{\circ}$ .

## 2.6. Paper chromatography.

For the separation of deoxyribonucleosides and ribonucleosides a two solvent system described by Sulkowski & Iaskowski (1962) was used. Solvent I consisted of 95% ethanol-1M ammonium acetate adjusted to pH 7.5 (70:35 v/v), solvent II was isopropanol-water-saturated ammonium sulphate (2:18:80 by vol.). Whatman No. 3mm paper was used, and both solvents were used in a descending direction. The development time for the first solvent was 16 hr. and in the second 12 hr. This combination separated all the deoxyribonucleosides and ribonucleosides except cytidine and uridine which overlapped. The proportion of the two pyrimidines was therefore estimated by comparing the spectra of the eluted spots with the spectra of known mixtures of cytidine and uridine.

A third solvent was used in the separation of nucleosides from dephosphorylated oligonucleotides. The solvent consisted of isopropanol-water-concentrated ammonia



(7:2:1. by vol.). The development time was 16 hr. and the solvent was used in a descending direction. Whatman No. 1 paper was used.

Spots were located by viewing the chromatogram with a low pressure mercury lamp filtered to remove visible light. The material in a spot was eluted by first cutting out the spot, attaching it to a paper wick, which was then immersed in 0.01N HCl. The eluted material was identified and estimated by recording the spectrum in a Cary Model 11 spectrophotometer and by utilising the data given in the publication 'Properties of the Nucleic Acid Derivatives.' by Calbiochem, Los Angeles, California (Table 11.).



**TABLE 11****Spectral Data of the Nucleosides**

Nucleoside	$\lambda_{\text{max}}$	$\epsilon_{\text{max}} \times 10^3$	$\lambda_{\text{min}}$	$\epsilon_{\text{min}} \times 10^3$	$\frac{250}{260}$	$\frac{280}{260}$
Ado	256	14.6	230	3.5	0.84	0.22
Cyd	280	13.4	241	1.7	0.45	2.10
Guo	256	12.2	228	2.4	0.94	0.70
Ino	248	12.2	223	3.4	1.68	0.25
Urd	262	10.1	230	2.05	0.74	0.35
dAdo	258	14.1	228	3.0	0.83	0.24
dCyd	280	13.2	241	1.5	0.43	2.16
dGuo	255	12.3	-	-	1.02	0.70
dIno	249	13.2	-	-	1.63	0.23
dThd	267	9.65	235	2.2	0.65	0.72

The data has been taken from 'Properties of the Nucleic Acid Derivatives' by Calbiochem, Los Angeles, California.



### 3. Results.

#### 3.1. Examination of the products of prolonged digestion.

Exhaustive digestion of the substrate was excluded because it was considered that under conditions to achieve exhaustive digestion of the substrate, the contaminating activities, that is phosphomonoesterase, phosphodiesterase I and II, would appreciably contribute to the result. For this reason digestion was reduced to a 12 hr. incubation. The digestion mixture contained 40 mg. ascites tumor cell denatured DNA, 1.5 mmoles imidazole-HCl buffer pH 6.8, 15  $\mu$ moles  $MgCl_2$ , 300  $\mu$ moles 2-mercaptoethanol in a volume of 20 ml. The solution was incubated at 37° for 12 hr. At zero time 90 units of the liver DNase were added followed by a further 90 units after 4 hr. and after 8 hr. On completing the incubation the solution was deproteinised by shaking with an equal volume of chloroform-isoamyl alcohol (4:1 v/v) for 1 hr. The aqueous layer was removed and it was diluted to 150 ml. with water. The solution contained 830 E<sub>260</sub> units. After the digest had been applied to the column of DEAE-cellulose, gradient elution was commenced. The gradient was obtained by using a two vessel open system containing 1.4 litres of 0.01M and 1.0M triethylammonium bicarbonate buffer pH 8.6. The flow rate of the column was 4 ml./min. and 16.5 ml. fractions were collected. At the end of the run samples



were removed from selected fractions for the determination of the gradient. The  $E_{260}$  was measured for each fraction (Fig. 16). 98% of the  $E_{260}$  units was recovered. The fractions were pooled according to the  $E_{260}$  values into fractions as indicated in Fig. 16. These seven fractions were freeze-dried until the characteristic smell of triethylamine could not be detected. The material was then dissolved in 4 ml. water and the  $E_{260}$  was measured.

The chain length of the pooled fractions was determined by comparing the total phosphorus with the end terminal phosphorus of an oligonucleotide fraction. Total phosphorus was determined as described in chapter 2 section 2.3.2. Approximately 8.0  $E_{260}$  units were used from each of the pooled fractions wherever this was possible. The estimation of the end terminal phosphorus was performed on an equal quantity of oligonucleotides as was used for the total phosphorus determination. The sample was incubated with 0.02 ml. alkaline phosphatase suspension and 50  $\mu$ moles tris-HCl buffer pH 8.0 in a volume of 2 ml. at 37° for 1 hr. The inorganic phosphorus released was estimated by the method of Allen (1940) as described in chapter 2 section 2.3.2.

The digestion products of the RNase activity on 40 mg. yeast RNA were examined in a similar manner (Fig. 17). The recovery of  $E_{260}$  units was 100%. The results of the determination of



Figure 16.

Fractionation of the products of digestion of DNA by the purified liver nuclease.

The digestion medium contained 40 mg. ascites carcinoma denatured DNA, 1.5 mmoles imidazole-HCl buffer pH 6.8, 15  $\mu$ moles  $MgCl_2$ , 300  $\mu$ moles 2-mercaptoethanol in a total volume of 20 ml. and was incubated at  $37^\circ$  for 12 hr. At zero time 90 units of the purified liver DNase were added and after 4 hr. and 8 hr. The gradient consisting of 1.4 litres 0.01M triethylammonium bicarbonate buffer (TEAB) pH 8.6 and 1.4 litres 1.0M of the same buffer was applied to a column, 2 x 40 cm., of DEAE-cellulose. The flow rate was 4 ml./min. and 16.5 ml. fractions were collected.



Figure 16.

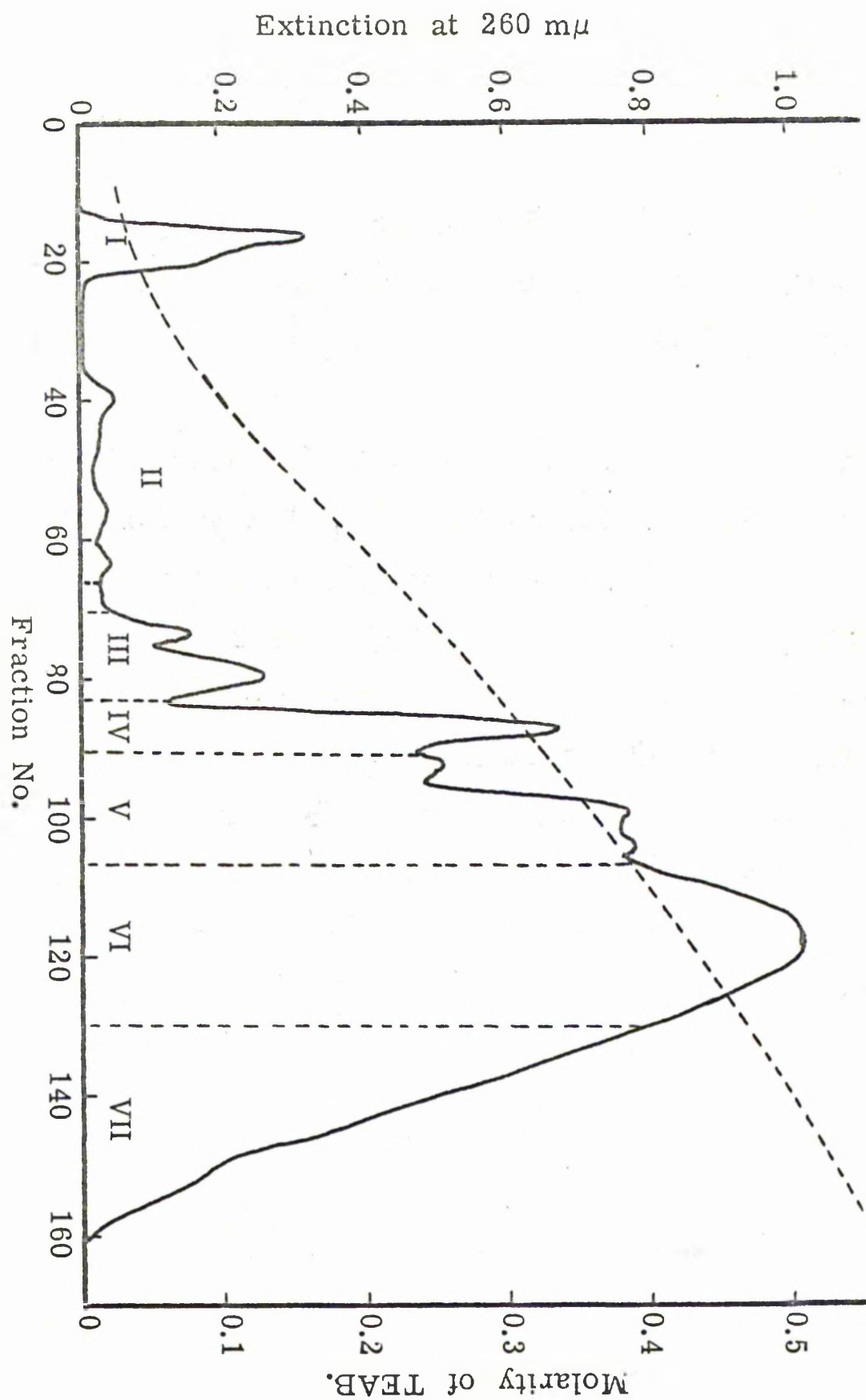




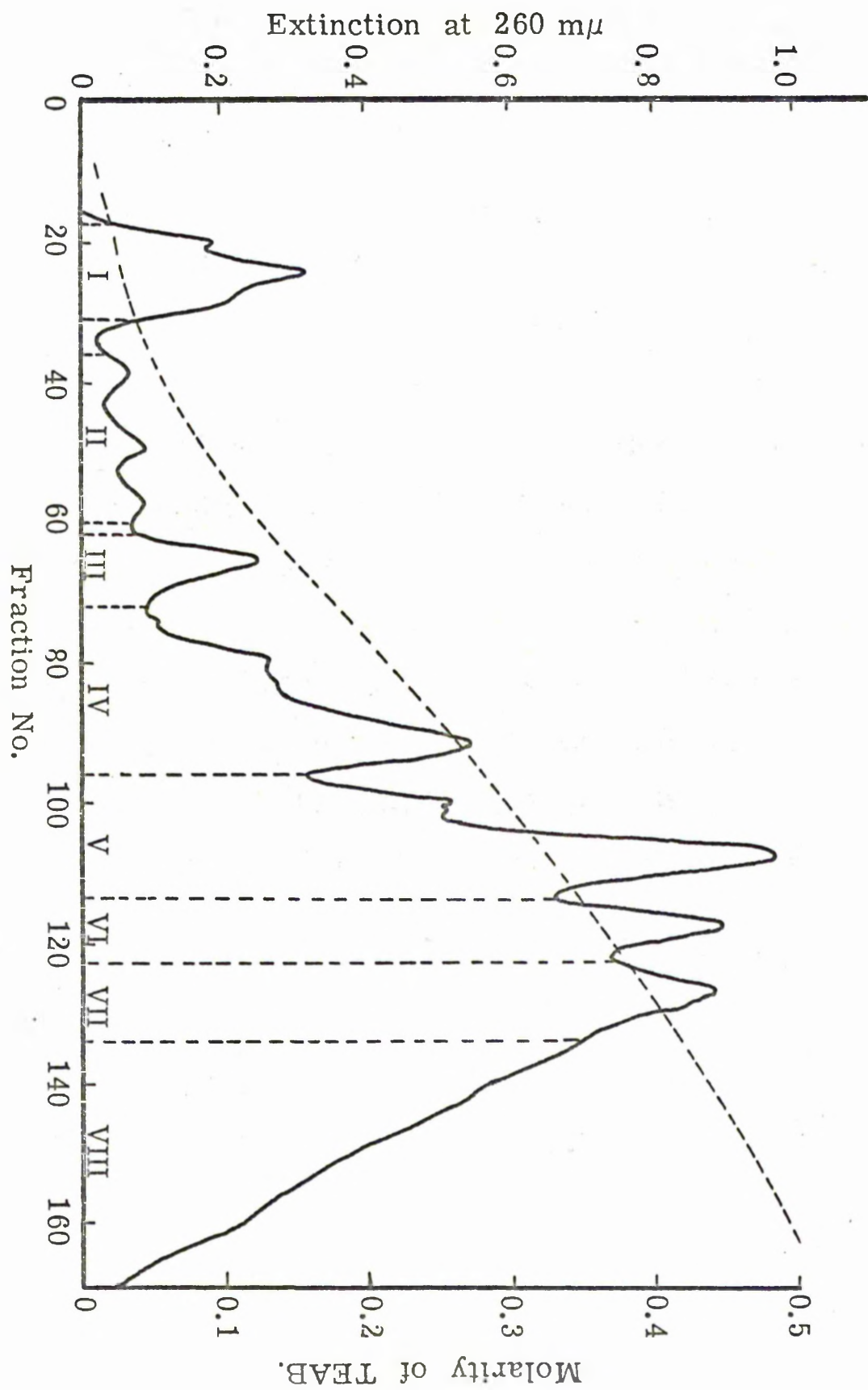
Figure 17.

Fractionation of the products of digestion of RNA by the purified liver nuclease.

The digestion medium contained 40 mg. yeast RNA, 1.5 mmoles imidazole-HCl buffer pH 6.8, 15  $\mu$ moles  $MgCl_2$ , 300  $\mu$ moles 2-mercaptoethanol in a total volume of 20 ml. and was incubated at 37° for 12 hr. At zero time 90 units of the purified liver RNase were added and after 4 hr. and 8 hr. The gradient consisting of 1.4 litres 0.01M triethylammonium bicarbonate buffer (TRAB) pH 8.6 and 1.4 litres 1.0M of the same buffer was applied to a column, 2 x 40 cm., of DEAE-cellulose. The flow rate was 4 ml./min. and 16.5 ml. fractions were collected.



Figure 17.





the chain lengths of the products are given in Table 12. To enable these results to be evaluated more accurately, the contaminating activities, phosphomonoesterase, phosphodiesterase I and II, of the enzyme solutions used in both digests were assayed as described in chapter 2 section 2.4.5.6 and 7. The values obtained were then calculated to give the amount of hydrolysis that would occur under the conditions of the prolonged digestion. It was found that for the DNA digest phosphomonoesterase would release 0.34  $\mu$ moles p-nitrophenol, phosphodiesterase II 0.29  $\mu$ moles, and phosphodiesterase I showed no activity. For the RNA digest phosphomonoesterase would release 0.21  $\mu$ moles p-nitrophenol, phosphodiesterase I and II 0.02  $\mu$ moles each.

The results indicate that both activities, DNase and RNase, are almost exclusively endonucleolytic in their mode of action.

### 3.2. Determination of the position of the end terminal phosphate

To determine the position of the end terminal phosphate use was made of certain properties of snake venom phosphodiesterase and spleen phosphodiesterase, namely the inability of snake venom phosphodiesterase to hydrolyse oligonucleotides terminated by a 3'-phosphate group, and the



**TABLE 12**

**The chain length of the products of digestion  
of liver nuclease**

Fraction		Terminal Phosphorus μmoles	Total Phosphorus μmoles	Chain Length	% of Total Digest
RNA					
I	( 18- 31)	0	0	0	5.7
II	( 36- 60)	0	0.28	1*	2.9
III	( 63- 72)	0.21	0.40	1.9	3.4
IV	( 73- 96)	0.38	0.94	2.5	13.1
V	( 97-114)	0.37	1.02	2.8	21.9
VI	(115-123)	0.18	0.53	2.9	13.1
VII	(124-134)	0.31	1.05	3.4	16.0
VIII	(134-170)	0.10	0.53	5.3	24.0
DNA					
I	( 15- 21)	0	0	0	4.3
II	( 37- 66)	0	0.17	1*	2.4
III	( 70- 83)	0.43	0.93	2.2	5.6
IV	( 84- 91)	0.37	1.02	2.7	9.4
V	( 92-107)	0.21	1.00	4.7	24.6
VI	(108-130)	0.16	0.98	6.1	57.0
VII	(131-160)	0.12	1.21	10.1	27.0

\* The position of this fraction in the DEAE-cellulose effluent and the presence of phosphorus in the  $H_2SO_4$  digest indicate that these fractions contain mononucleotides though no phosphorus was released by alkaline phosphatase.



inability of spleen phosphodiesterase to hydrolyse oligonucleotides terminated by a 5'-phosphate group. Therefore the digestion of oligodeoxyribonucleotide products of the liver DNase by snake venom phosphodiesterase and spleen phosphodiesterase was compared with the corresponding digestion of dephosphorylated oligodeoxyribonucleotides. Similarly the digestion of oligoribonucleotide products of the liver RNase was compared with the digestion of dephosphorylated oligoribonucleotides.

Dephosphorylated oligodeoxyribonucleotides were prepared by the digestion of 4 mg. ascites tumor cell DNA with 200 µg. pancreatic DNase in the presence of 200 µmoles tris-HCl buffer pH 7.5 and 100 µmoles MgCl<sub>2</sub> in a total volume of 5 ml. at 37° for 1 hr. The pH of the solution was then adjusted to 8.0 and 0.01 ml. alkaline phosphatase suspension was added. The solution was incubated at 37° for a further 1 hr. The phosphatase was inactivated as described in this chapter section 2.4. The solution contained approximately 100 K<sub>2</sub>60 units. Dephosphorylated oligoribonucleotides were prepared similarly by the digestion of 4 mg. yeast with 200 µg. pancreatic RNase. The oligodeoxyribonucleotide products of the liver DNase were prepared by incubating 20 mg. salmon sperm denatured DNA with 250 µmoles imidazole-HCl buffer pH 6.8, 5 µmoles MgCl<sub>2</sub>, 100 µmoles 2-mercaptoethanol and 50 units



of the purified liver RNase in a total volume of 10 ml. at 37° for 4 hr. 1 ml. of this solution containing 50 R<sub>260</sub> units was used in this experiment. The remaining 9 ml. were used in the succeeding experiment. Oligoribonucleotides were prepared similarly by digesting 20 mg. yeast RNA with 70 units of the purified liver RNase.

The solutions of the dephosphorylated oligodeoxyribonucleotides and the oligodeoxyribonucleotide products of the enzyme were each divided into three fractions. The first fraction was set aside as a control to give a measure of mononucleotides released in the preliminary digestion. The pH of the second fraction was adjusted to 6.5 and 200 µg. spleen phosphodiesterase was added. The solution was incubated at 37° for 1 hr. The reaction was stopped by heating at 100° for 10 min. The pH of the third fraction was adjusted to pH 8.8 and 0.1 ml. of the purified snake venom phosphodiesterase was added. The solution was incubated at 37° for 1 hr. The reaction was stopped by heating at 100° for 10 min. The dephosphorylated oligoribonucleotides and the oligoribonucleotide products of the liver RNase were treated similarly.

The amount of mononucleotides produced in each fraction was determined chromatographically. To facilitate the separation of the mononucleotides from all other products each



fraction was dephosphorylated. The pH of each fraction was adjusted to 8.0 and 0.01 ml. alkaline phosphatase suspension was added. The fractions were incubated at 37° for 30 min. The fractions then were concentrated by freeze-drying and the material was redissolved in 0.05 ml. 0.01N HCl. 0.03 ml. of each fraction was spotted on Whatman No. 1 paper and the chromatogram was developed in a descending direction in isopropanol-water-concentrated ammonia (7:2:1 by vol.) for 16 hr. The nucleosides were separated completely from all the other products. The material corresponding in  $R_f$  to the four known nucleosides added as markers was eluted and the  $E_{260}$  value measured (Table 13). The results show that in comparison with the dephosphorylated oligonucleotides, the oligodeoxyribonucleotides and oligoribonucleotides were both relatively resistant to spleen phosphodiesterase but both were hydrolysed by snake venom phosphodiesterase. The DNase and RNase activity of the purified liver nuclease would both appear to produce oligonucleotides terminated by 5'-phosphate groups.

### 3.3. Determination of the terminal bases of the oligonucleotides

The terminal bases of the oligonucleotide products on digestion of DNA and RNA with the purified liver nuclease may



**TABLE 13**

**The digestion of the oligonucleotide products  
of the liver nuclease with spleen and snake  
venom phosphodiesterase**

Fraction	$E_{260}$ of the nucleosides released by	
	Spleen	S.V.
DNA products		
1. Dephosphorylated oligonucleotides	0.44	2.16
2. Oligonucleotide products of the liver nuclease	0.01	1.01
RNA products		
1. Dephosphorylated oligonucleotides	0.34	0.49
2. Oligonucleotide products of the liver nuclease	0.08	0.64



be examined by first dephosphorylating the oligonucleotides. The dephosphorylated oligonucleotides were digested with snake venom phosphodiesterase, which removed 5'-nucleotides starting from the 3' end of the oligonucleotide. The terminal base therefore was released as a nucleoside. Digestion with spleen phosphodiesterase similarly released the base at the 3' end of the oligonucleotide as a nucleoside.

The oligodeoxyribonucleotides and oligoribonucleotides were prepared as previously described (this chapter section 3.2.), the remaining 9 ml. of the digest being used. The two solutions of oligonucleotides were dephosphorylated by adjusting the pH of each solution to 8.0 and adding 0.02 ml. alkaline phosphatase suspension. The solutions were incubated at 37° for 1 hr. The alkaline phosphatase was inactivated and the two solutions were divided into two. One half was adjusted to pH 8.8 and 0.5 ml. snake venom phosphodiesterase was added, and the solution was incubated at 37° for 4 hr. The other half was adjusted to pH 6.5 and 1 mg. spleen phosphodiesterase was added. The solution was incubated at 37° for 6 hr. and the extent of the digestion was measured by comparing the total phosphorus of a sample with the inorganic phosphorus released by incubation with alkaline phosphatase. The assay showed that the digestion with snake venom phosphodiesterase had proceeded to completion,



but with spleen phosphodiesterase digestion of the oligodeoxyribonucleotides and oligoribonucleotides had proceeded only 60% and 66% respectively. Incubation of these solutions was therefore continued for a further 2 hr. The four digests were freeze-dried and redissolved in 0.1 ml. 0.01N HCl.

The nucleosides were resolved by 2-dimensional paper chromatography using the solvent systems of Sulkowski & Laskowski (1962). Figures 18. and 19. illustrate the separation obtained with known nucleosides. The dotted outline in these figures indicates the position of a spot obtained with all four digests, and whose spectrum indicated that it was not a purine or pyrimidine base. These facts suggested that its origin was in the reagents used in the digestion other than DNA and RNA, in particular 2-mercaptoethanol. 2-mercaptoethanol was therefore chromatographed as above. It gave an identically positioned spot, whose spectrum on elution was similar to the above mentioned spot consisting of a slow increase in the extinction from 280 to 240 mμ when the extinction rapidly increased. Apart from this spot digestion with snake venom phosphodiesterase gave four spots corresponding in their position to dAdo, dGuo, dCyd and dThd, and Ado, Guo, Cyd and Urd. With digestion with spleen phosphodiesterase five



Figure 18.

Chromatographic separation of deoxyribonucleosides and 5'-deoxyribonucleotides.

Solvent 1 consisted of 95% ethanol-1M ammonium acetate pH 7.5 ( 70:35 v/v ). Solvent 2 consisted of isopropanol-water-saturated ammonium sulphate ( 2:18:80 by volume ). The chromatogram was developed in solvent 1 for 16 hr., and in solvent 2 for 12 hr. both in a descending direction. The dotted outline indicates the position of 2-mercapto-ethanol.



Figure 18.

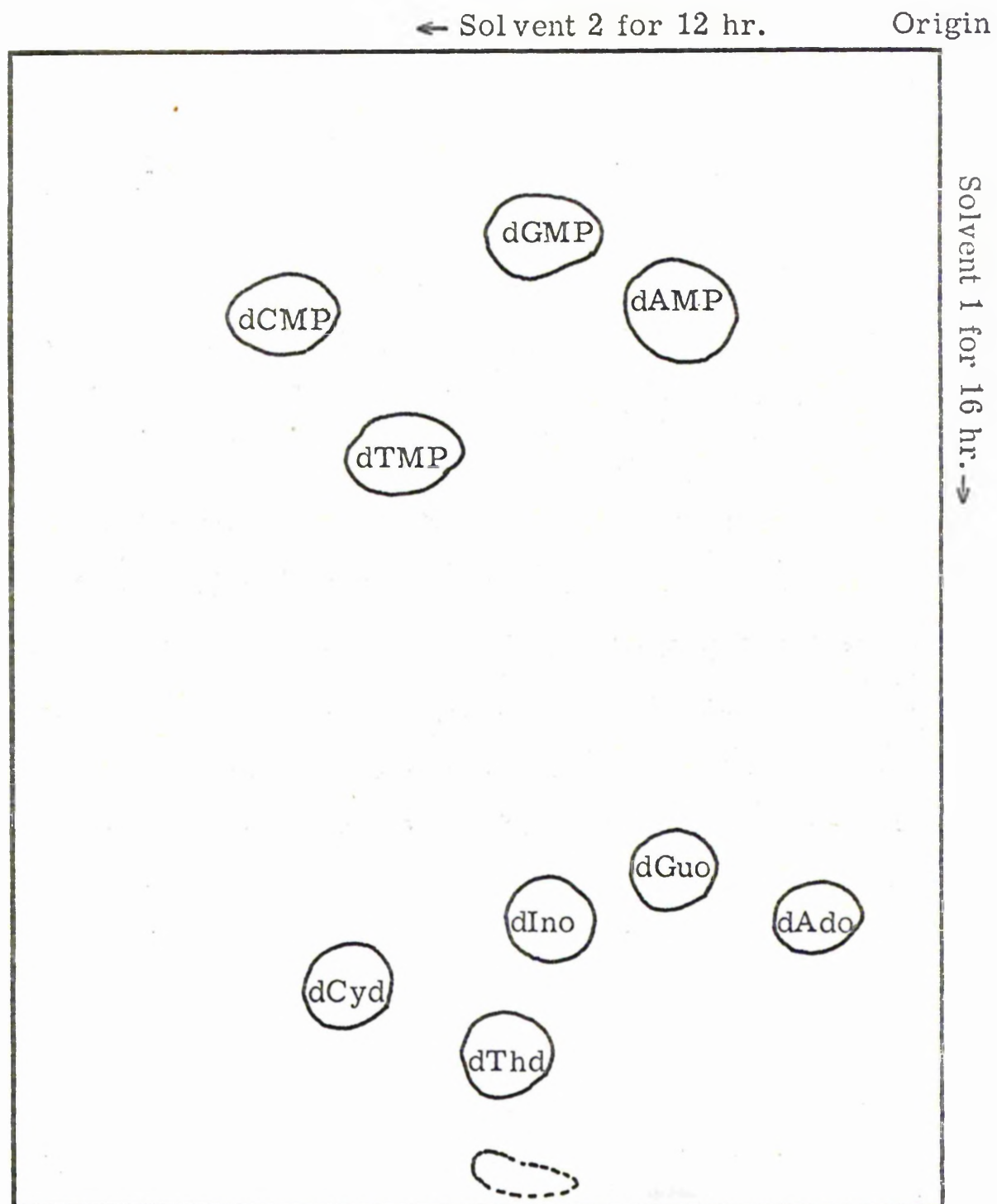




Figure 19.

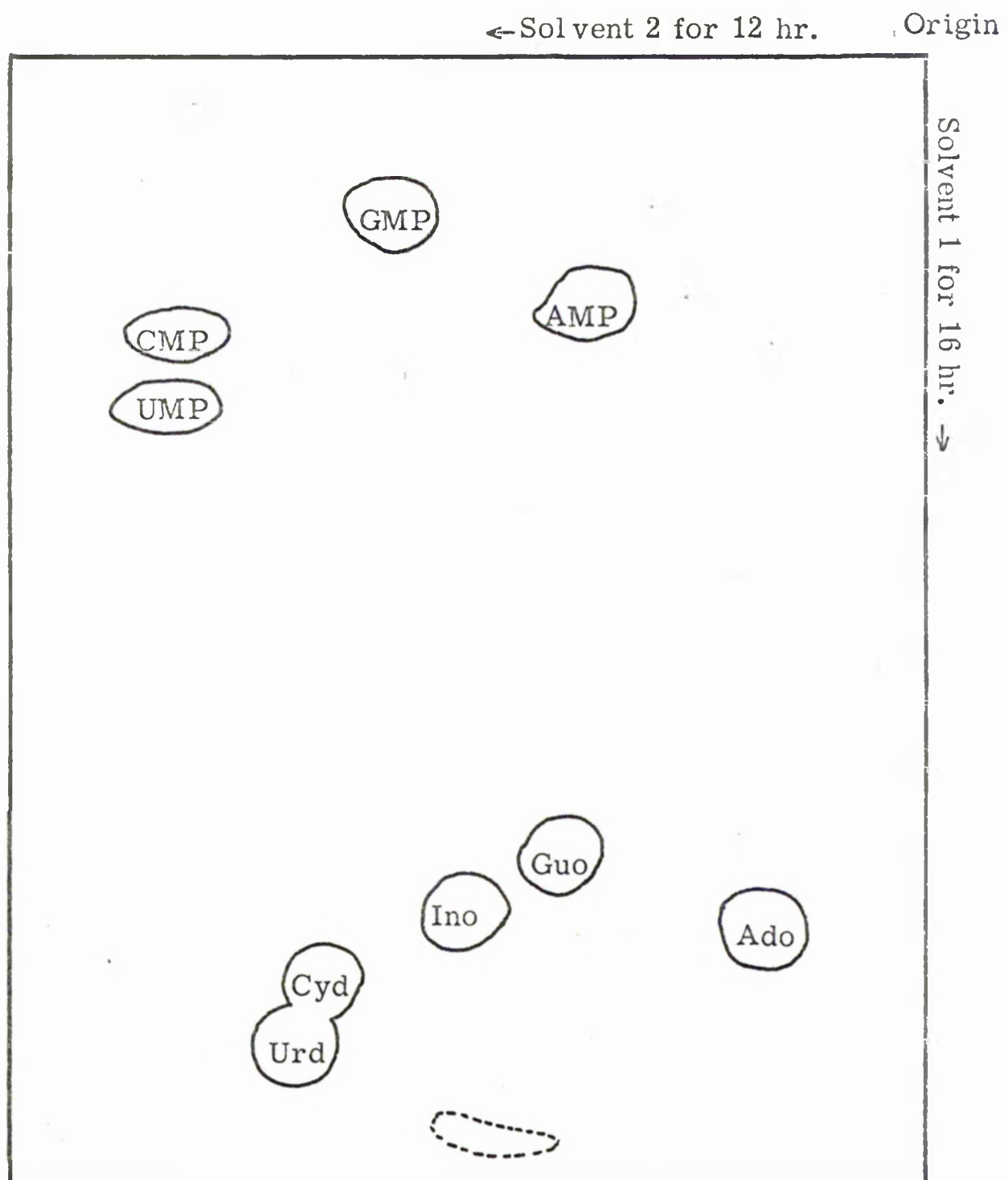
Chromatographic separation of ribonucleosides and 5'-ribonucleotides.

The solvents were the same as those used in the separation of the deoxyribonucleosides, and the development of the chromatogram was identical.

The dotted outline again indicates the position of 2-mercaptoethanol.



Figure 19.





spots were obtained corresponding to dAdo, dGuo, dIno, dCyd and dThd, and Ado, Guo, Ino, Cyd and Urd. The additional spots corresponding to Ino and dIno had been expected, for a paper by Winter & Bernheimer (1964) had shown that the commercial preparation of spleen phosphodiesterase contained a deaminase specific for Ado and dAdo that gave Ino and dIno respectively. The spleen phosphodiesterase used in these studies was therefore incubated with Ado and dAdo under conditions similar to those used in the digestion of the oligonucleotides. Under these conditions the enzyme deaminated 15  $\mu$ moles of Ado and dAdo completely to give Ino and dIno respectively.

The eluted nucleosides were estimated by recording their spectra and using the molar extinction coefficients quoted in the publication 'Properties of the Nucleic Acid Derivatives' by Calbiochem, Los Angeles, California. The results (Table 14.) showed that the two activities DNase and RNase have no marked preferences towards the bases.

To check that the procedures adopted were suitable, the terminal bases of oligonucleotides produced by digestion of RNA with pancreatic RNase were determined in an exactly similar manner. The results (Table 15.) illustrate the known specificity of pancreatic RNase for pyrimidine bases. The adenosine and inosine obtained may reflect the presence



TABLE 14

The end terminal nucleosides of the oligonucleotide products of the liver nuclease, expressed as percentage of the total amount of nucleosides released.

Nucleoside	% of total nucleoside at 5' position	% of total nucleoside at 3' position	% composition of substrate
<u>RNase</u>			Yeast RNA <sup>1</sup>
Ado	36	46.5	25.0
Cyd	9.5	8.5	20.1
Guo	33.5	30.5	30.2
Urd	22	15	24.6
<u>DNase</u>			S.S. DNA <sup>2</sup>
dAdo	30	42	29
dCyd	17	27	20.8
dGuo	25	14	20.4
dThd	28	15	29.1

<sup>1</sup> Values for yeast RNA were taken from Magasanik (1955).

<sup>2</sup> Values for salmon sperm DNA were taken from Chargaff (1955).



**TABLE 15**

The end terminal nucleosides of the oligonucleotide products of pancreatic RNase, expressed as percentages of the total amount of nucleosides released.

Nucleoside	% of total nucleoside at 5' position	% of total nucleoside at 3' position
Ado	19.5	7
Cyd	20.0	33
Guo	20.5	0
Urd	40.0	61



of phosphatase in the spleen phosphodiesterase or the remnants of the alkaline phosphatase that had previously been added. However the results indicate that the procedure used could be expected to give a reasonable estimation of the proportion of the bases at the 3' and 5' ends of the digestion products of the liver nuclease.



#### 4. Discussion.

The results indicate that the activity of the liver nuclease towards both DNA and RNA is endonucleolytic and results in the production of oligonucleotides terminated by 5'-phosphate groups. The activity shows no specificity towards the bases.

The quantity of nucleosides plus mononucleotides produced was greater than the assays for phosphodiesterase I and II suggested, assuming that the nucleosides were the products of hydrolysis of mononucleotides by phosphomonoesterase activity. However the amount obtained might readily have arisen from the hydrolysis of, for example, pentanucleotides to give tetranucleotides and mononucleotides, the activities still being endonucleolytic in nature. The higher proportion of longer oligonucleotides in the digestion of DNA in comparison with those produced in the digestion of RNA may reflect a fundamental difference in the mechanism of action of DNase and RNase. Alternatively the result may be a reflection of the greater stability of the RNase compared with that of the DNase and also the smaller size of the substrate for the RNase compared with the substrate of DNase.

The position of the terminal phosphate of the oligodeoxy-ribonucleotides produced by the action of the liver DNase is clearly indicated as the 5' position for the activity of the



snake venom phosphodiesterase is almost completely inhibited by the presence of a 3'-phosphate group. However with oligoribonucleotides there is an additional possibility to consider, namely the possibility of the formation of 2',3'-cyclic phosphate groups. In the presence of such groups snake venom phosphodiesterase would be expected to hydrolyse such oligoribonucleotides, though at a rate lower than the rate of hydrolysis of dephosphorylated oligoribonucleotides. However the results were contrary to this possibility. In addition, oligoribonucleotides terminated by a 2',3'-cyclic phosphate group would be readily hydrolysed by spleen phosphodiesterase. Therefore the only interpretation of the results is that the liver RNase produces oligonucleotides terminated by 5'-phosphate groups.

The studies conducted to determine the specificity of the DNase and RNase indicate that there is no marked specificity towards the bases with either of the activities. If the percentages of the four nucleosides occurring at the terminal positions of the oligonucleotides are compared with the percentage base composition of the substrate used, the results compare reasonably with these values except for adenosine and deoxyadenosine which occur at a higher percentage in the terminal position than would be expected from their distribution in the substrate. However such a result may



only indicate that the bonds linking adenosine and deoxyadenosine to the phosphate groups are slightly more labile to hydrolysis than the remaining linkages rather than the enzyme's affinity for those bases.

The results may then be viewed in a negative manner, in that no evidence was obtained to indicate that the activities of the purified liver nuclease towards DNA and RNA belong to different proteins.



# 1. Characteristics of the liver nuclease.

From the available evidence the enzyme, partially purified from rat liver mitochondria, appears to hydrolyse both DNA and RNA. The two activities of the enzyme are very similar in every respect except in their storage capabilities. Thus the enzyme hydrolyses DNA and RNA endonucleolytically producing oligonucleotides terminated by 5'-phosphate groups. The enzyme shows no preference towards any one of the four bases. The pH optimum for both activities is about 6.8 - 7.0, and the enzyme shows an absolute requirement for  $Mg^{2+}$  or  $Mn^{2+}$  ions.  $Ca^{2+}$  ions inhibit the enzyme as also do  $Na^{+}$  ions. The enzyme is readily denatured by heating, the two activities being lost at equivalent rates.

The DNase activity of the liver nuclease is clearly distinct from that of DNase type II in its pH optimum, ionic requirements, products of hydrolysis and its subcellular localisation. It is basically similar to pancreatic DNase I. The pH optima are similar and the requirement for  $Mg^{2+}$  is of the same order;  $Na^{+}$  ions inhibit at approximately 0.05M and the products of hydrolysis are similar. However there are a few important differences.  $Ca^{2+}$  ions alone stimulate pancreatic DNase I weakly but in the presence of  $Mg^{2+}$  there is a powerful synergistic effect (Wiberg, 1958), the maximum effect occurring when  $Ca^{2+}/Mg^{2+}$  is 10:1. In contrast the



CHAPTER IV  
General Discussion.



liver nuclease is inhibited by  $\text{Ca}^{2+}$  ions. However this difference may not be as marked, for Shack & Bynum (1964) failed to show a synergistic effect of  $\text{Ca}^{2+}$  with  $\text{Mg}^{2+}$  under optimum conditions with pancreatic DNase I. The second point of difference is related to the effect of the secondary structure of the substrate. Pancreatic DNase I hydrolyses native DNA several times more rapidly than denatured DNA whereas the liver DNase shows a preference for denatured DNA. In this respect the liver DNase resembles more closely the endonuclease from lamb brain (Healey et al., 1963). The endonuclease from lamb brain shows however an absolute requirement for denatured DNA. But the two activities appear to have very similar properties. They respond to heat, freezing and freeze-drying similarly and 2-mercaptoethanol and glycerol both effectively stabilise the enzymes when they are stored at  $0^{\circ}$ . In addition, the purified lamb brain endonuclease contains RNase activity (Burdon, personal communication), though whether this activity belongs to the same protein as the DNase activity has yet to be determined.

There are very few enzymes that hydrolyse RNA endonucleolytically to produce oligonucleotides terminated in 5'-phosphate groups. Spahr (1964) has partially purified a RNase from E. coli that has an alkaline pH optimum and a requirement for  $\text{Mg}^{2+}$ . However this enzyme hydrolyses RNA



exonucleolytically as well as endonucleolytically. From mammalian tissues (liver nuclei), Heppel et al. (1956) have obtained an enzyme that hydrolyses poly A readily to produce oligonucleotides terminated by 5'-phosphate groups at an alkaline pH optimum and its activity shows a requirement for  $Mg^{2+}$ .

However, if the two activities of the liver nuclease described in this thesis belong to one protein, as the evidence strongly suggests, the enzyme should more correctly be compared with the other known enzymes that hydrolyse both DNA and RNA endonucleolytically. Two enzymes are known with these properties. One enzyme purified from mung bean sprouts (Sung & Laskowski, 1962) has a pH optimum of 5.0. This enzyme shows a requirement for  $Mg^{2+}$ , and has a partial preference for -pApX-linkages. The other enzyme, partially purified from Azotobacter agilis (Stevens & Hilmeo, 1960) has an alkaline pH optimum and a requirement for  $Mg^{2+}$  ions.

## 2. The nucleases of the liver.

To understand the role of the enzyme partially purified from rat liver mitochondria it is necessary first to describe the other enzymes involved in the degradation of nucleic acids in the liver.

Swingle & Cole (1964) have described an acid DNase activity



in the nuclei of rat liver. To detect such an activity it was found necessary to isolate the nuclei in the presence of  $\text{Ca}^{2+}$  ions. In the absence of  $\text{Ca}^{2+}$ , no activity could be detected, the activity presumably leaking out of the nuclei. The activity was shown not to be due to cosedimentation of unbroken cells or lysosomes or precipitation of a soluble DNase. In addition there is an alkaline RNase that hydrolyses RNA producing oligonucleotides terminated by 5'-phosphate groups. (Heppel et al., 1956). This enzyme will be termed alkaline RNase I to distinguish it from the other alkaline RNases in rat liver. Roth (1957) has partially purified an alkaline RNase, from rat liver mitochondria, termed RNase II, that resembles closely pancreatic RNase. It has an pH optimum about 7.0, no requirement for  $\text{Mg}^{2+}$ , and for optimum activity requires 0.1M NaCl. In addition it is stable to heat, and its products of hydrolysis include a resistant 'core', and oligonucleotides terminated by pyrimidine 2',3'-cyclic phosphates. The cyclic phosphates are probably hydrolysed slowly as Beard & Razzell (1964) have shown that there is an inhibitor of this step in the purified enzyme. Also there is the enzyme characterised in this thesis that hydrolyses DNA and RNA, and appears to be located in the mitochondria. Within the lysosomes there are two endonucleases, termed acid DNase and acid RNase. The acid



DNase though it has not been characterised with respect to its products or hydrolysis is presumably similar to the acid DNase purified from spleen (Koerner & Sinsheimer, 1957) as it has a pH optimum about 5 and shows no requirement for  $Mg^{2+}$ . The acid RNase has been characterised by Roth (1957) and Haver & Greco (1962). It was shown to have a pH optimum of 5.4 and no requirement for  $Mg^{2+}$ . It differs from alkaline RNase II with respect to its products on hydrolysis of RNA. This enzyme produces purine 2',3'-cyclic phosphates as well as pyrimidine 2',3'-cyclic phosphates, and no undigestible 'core' is produced. It also differs from alkaline RNase II with respect to its heat stability which is considerably less than alkaline RNase II. The microsome fraction contains a RNase (Tashiro, 1958) though it has not been characterised. However the RNase purified from E. coli ribosomes was shown to be similar to pancreatic RNase (Spahr & Hollingsworth, 1961). In addition there is a phosphodiesterase type I that hydrolyses both DNA and RNA producing 5'-mononucleotides (Razzell, 1961). In the supernatant liquid there is a phosphodiesterase type II, which however has been shown to be present in the other subcellular fractions. The question of whether there is endonuclease activity in the supernatant liquid is difficult to answer because of the presence of inhibitors of DNase and RNase. Roth (1958) has



characterised an inhibitor of alkaline RNase II from the supernatant liquid; it does not inhibit acid RNase. The DNase inhibitor acts on pancreatic DNase and DNase from rat liver mitochondria (Loiselle & Carrier, 1963; Zalite & Roth, 1964). Therefore there are probably no active nucleases in the cytoplasm of the liver cells. However Reid & Nodess (1963) presented evidence that suggested the presence of an acid DNase in the supernatant fluid from cell homogenates.

### 3. The functions of the nucleases.

The nuclease activities of the nuclei, microsomes and cell sap are illdefined and incompletely characterised so that a meaningful discussion of these activities is not possible. Far more is known of the activities within the mitochondria and the lysosomes and also of the particles themselves.

#### 3.1. The lysosomal enzymes.

The evidence of Beaufray et al. (1959) suggested that several acid hydrolases including acid DNase and acid RNase were confined within the same particles, so that disruption of the particles released all of the enzymes together. Therefore in considering the functions of acid DNase and acid RNase it is sufficient and justifiable to consider the functions of the lysosomes.



An indication of the role played by lysosomes within a cell comes from the study of phagocytes found in the blood. Certain groups of the phagocytes contain numerous granules as seen under the light microscope. Isolation of these granules has shown that they contained many of the acid hydrolases present in the lysosomes of the liver. The function of phagocytes in the blood is to engulf and digest foreign material, for example bacteria, that may enter the blood stream. Morphological studies of the phagocytes showed that the cytoplasmic granules disappeared following the ingestion of microorganisms by the phagocytes (Hirsch & Cohn, 1964). It was inferred from these observations that the cytoplasmic granules were involved in the digestion of the organisms. However the question of how the contents of the lysosomes are transferred to the vacuole has not been investigated. It might be assumed that the membranes of the two vacuoles fuse, and the acid hydrolases would enter directly into the vacuole. Release of these enzymes into the cytoplasm would probably lead to the death of the cell. It was observed that following the lysis of lysosomes by such agents as detergents the cytoplasm of the cells liquified. Viruses may similarly enter cells by pinocytosis. It might be expected that the vacuoles containing viruses would be treated in a similar manner to microorganisms ingested by phagocytes. In this



way the viral coat would be digested. For viral infection of the cell to occur it is obviously necessary for the nucleic acid to be protected, and there are two possibilities. The nucleic acid may exist as a resistant nucleoprotein, or the virus may contain an inhibitor of acid DNase or acid RNase. This second possibility is suggested by the results of Tunis & Regelson (1963) and Bernardi (1964). They showed that acid DNase was inhibited by polyanionic compounds, which included RNA.

Thus one would expect that lysosomes would be associated with cells capable of pinocytosis. A tissue where pinocytosis fulfills an important function is the intestinal mucosa for digestion of food particles probably occurs intracellularly as well as extracellularly. The cells lining the intestinal lumen ingest food particles by pinocytosis and the particles would subsequently be digested by the lysosomal enzymes.

A second important function of the lysosomes may involve the autolysis of cells. There are two possible reasons why autolysis would be necessary. Where injury occurs to a tissue, by a physical or chemical agent, probably the first process that must occur before healing can begin would involve digestion of the damaged cells. In the damaged cells the lysosomal enzymes would presumably be released. However damaged cells may arise by mutations. Point mutations will



not usually affect a cell, for only a small percentage of the chromosome is functional. But mitotic aberrations may result in losses of a large proportion of the chromosome complement of a cell. It would therefore be to an animal's advantage to have some means of removing the deficient cells as accumulation of such cells in a tissue would decrease the efficiency of that tissue. Lysosomes would be able to fulfil this role.

The lysosomal enzymes may be involved in an animal's defence against starvation. When an animal is starving, the amino acids necessary for the synthesis of essential proteins are obtained by digestion of non-essential protein. The liver is a tissue rich in proteins, and probably is a major site of protein breakdown during starvation. The process may involve the formation of autophagic vacuoles (Novikoff, Essner & Quintana, 1964; Swift & Hruban, 1964). There are two possible ways that autophagic vacuoles may arise. One would involve extension of the endoplasmic reticulum so that it surrounded a large fraction of the cytoplasm which was eventually separated completely from the remaining cytoplasm by the membranes of the endoplasmic reticulum. Lysosomal enzymes would then enter the vacuole and digest its contents. The products would then diffuse out of the vacuole and be reutilised. The second possible process for the formation of



autophagic vacuoles would involve the separation of a section of the cytoplasm by pinocytosis followed by ingestion of this section. In both processes the result would be the same, that is a section of the cytoplasm separated by a membrane from the remainder of the cell.

These degradative functions of the lysosomes are then to be related to the findings of Brody & Balis (1959) and Goutier & Leonard (1962) that acid DNase increases in regenerating rat liver. The increase in acid DNase occurs at about 12 hr. after the operation, and maximum activity is reached at 36-41 hr. After 72 hr. the activity is still higher than in the normal liver. In contrast the maximum rate of DNA synthesis occurs at 39-44 hr. (Brody & Balis, 1959). Such results suggested to the authors that the increase in acid DNase was related to DNA synthesis. However a better explanation may be found by examining the paper of Coleman (1963), who demonstrated that acid DNase increased in the tail of Rana pipiens tadpole during metamorphosis, and was therefore involved in the resorption of the tadpole's tail. The results from the regenerating rat liver and those of Solomon (1964) who showed an increase of acid DNase in developing mouse embryo may be more accurately explained in terms of the previously suggested functions of lysosomes. Thus during growth and differentiation of the tissues many deficient cells may arise because of somatic mutations.



Autolysis of such cells could occur. Alternatively the lysosomes may play a more direct role in the growth of cells. Pinocytosis in the more simple animals, such as Amoeba, provides a means of transporting food material comparatively quickly from the exterior to the interior of a cell. Many animal cells retain the ability to form vacuoles and when rapidly dividing and growing pinocytosis may be involved in meeting the requirement for building materials of the cells. The contents of the vacuoles would then be digested by lysosomal enzymes and the products would be more readily available than materials circulating in the blood.

A relationship between RNase and growth has been demonstrated in plants (Ledoux, Galand & Huark, 1962). Such a relationship may depend on the necessity for the redistribution of macromolecules in, for example, the seedling of the plant.

### 3.2. The mitochondrial nucleases.

In contrast to the lysosomal enzymes the mitochondrial enzymes fulfill no obvious function. An indication of such functions may be obtained by an investigation of (i) the distribution of the nucleases in the different animal tissues and in the same tissues of a number of representative animals (ii) the location of the nucleases within the mitochondria, that is whether free in the sap of the mitochondria, bound



internally or externally, or an integral part of the membrane.

If the nucleases were bound internally or free in the sap of the mitochondria, their presence may be related to the occurrence of DNA in mitochondria (Nass & Nass, 1963; Kalf, 1964). The DNase may then remove DNA in the mitochondria which was unnecessary for expression of the mitochondrial functions. However this would depend on what DNA entered the mitochondria initially, that is whether any non-essential DNA appeared. The RNase activities may then be involved in the turnover of messenger RNA coded from the DNA. Alternatively the nucleases may be present to protect the DNA-primed RNA polymerase and the protein synthesising systems, that Kalf (1964) and Luck & Reich (1964) have shown to be present in mitochondria of lamb heart and N. crassa, from extraneous nucleic acids. However Nass & Nass (1964) have indicated that DNA appears in the mitochondria of many different tissues, while Shack (1958) showed that alkaline DNase was present in liver and kidney but effectively absent in spleen, thymus and lymphoma.

If the nucleases were bound externally to the mitochondria then presumably their function would lie in the cytoplasm. The nucleases may function as a means of curtailing synthesis of nucleic acids in the cytoplasm, while the inhibitors normally prevent any interference by the



nucleases. There are two possible situations where curtailment would be necessary. In viral infection the coat of the virus is removed when the virus enters the cell. For infection to occur the viral nucleic acid must be replicated. If this process were to occur in the cytoplasm the nucleases might act to prevent the redirection of the cells' synthetic systems to the replication of the virus. Alternatively DNA may appear in the cytoplasm derived from the chromosomes, since breakage of the arms of the chromosomes is known to occur during mitosis. Such pieces if they are not attached to the mitotic spindle are left in the cytoplasm when the nuclear membrane reforms. The DNA molecules if undigested might conceivably be replicated several times over since there is apparently a DNA polymerase in the cytoplasm. These molecules could further interfere with the cells metabolism by binding to the ribosomes possibly in a permanent manner (Takanami & Okamoto, 1963).

There is however a final consideration that is worth repeating, that is the possibility that enzymes purified from mitochondria do not show the same activity as they exhibit in the native state. The change in activity might be due to disaggregation of a macromolecular complex of which the purified activities were a part, or due to the alteration of the physical structure of the original protein.



## **SUMMARY.**



Summary.

1. Procedures for the purification of an alkaline DNase, showing a preference for denatured DNA, from rat liver were studied. Subcellular fractionation of rat liver showed that the nuclease was located largely in the mitochondria, and therefore mitochondria were used as the source of the enzyme. The enzyme was solubilised by treating the mitochondria with a hypotonic solution containing 0.1% triton-X-100.
2. The solubilised enzyme was purified further by ammonium sulphate fractionation, the fraction precipitating between 35-55% saturation being collected. This fraction was further purified by acetone precipitation, the fraction obtained between 36.9-46.9% acetone concentration being taken for further purification.
3. Column chromatography provided additional methods for purification of the enzyme. Passage of the dissolved acetone precipitate through a column of DEAE-cellulose equilibrated with 0.01M tris-HCl buffer pH 8.0 resulted in a further 10 fold increase in the specific activity of the enzyme. While 95% of the inactive protein was adsorbed to the cellulose, the enzyme was not. Gel filtration on Sephadex G-75 of the



effluent from DEAE-cellulose after concentration by precipitation with ammonium sulphate provided a further 4 fold increase in the specific activity.

4. The final product obtained from the livers of 10 rats contains approximately 0.4 mg. protein and 200 DNase units and represents a 715 fold increase in the specific activity. The purified enzyme contains negligible amounts of acid DNase, phosphatase and phosphodiesterase. However the enzyme hydrolyses native DNA at a lower rate and RNA at a higher rate than denatured DNA. These activities appear to be properties of the same protein since the three activities are eluted in identical positions on filtration through a column of Sephadex G-75 and G-200. In addition the ratio of the activity towards denatured DNA and native DNA was approximately the same as the ratio of the activity towards highly polymerised RNA and rRNA.

5. The enzyme could not be stored in ice, frozen or freeze-dried without loss of activity. However it was found that in the presence of 20% glycerol or 0.01M 2-mercaptoethanol the enzyme retained activity for upto 2 weeks. In these experiments however the activity towards denatured DNA decreased more rapidly than the activity towards RNA, suggesting that the two activities belong to separate proteins.



6. The molecular weight of the enzyme was estimated by comparing its elution volume for Sephadex G-75 with those of proteins of known molecular weight. The value obtained was approximately 44,000.

7. The activities of the enzyme towards denatured DNA and RNA were examined. Hydrolysis of the substrates was linear with respect to time upto about 50% acid-solubilisation of the substrates. There was a linear relation between enzyme concentration and the rate of hydrolysis for RNase and for DNase after a lag period at low enzyme concentrations. The Michaelis constants for both substrates were approximately the same, about  $8 \times 10^{-5}M$ . The two activities showed a sharp pH optimum about 6.8 - 7.0, and an absolute requirement for  $Mg^{2+}$  or  $Mn^{2+}$ , the optimal concentration for both  $Mg^{2+}$  and  $Mn^{2+}$  being about  $10^{-4}M$ . No difference was observed when DNA or RNA were used as substrates.  $Ca^{2+}$  showed no stimulatory effect and in the presence of the optimal concentration of  $Mg^{2+}$  50% inhibition of DNase and RNase was observed at  $Ca^{2+}$  concentrations of 3.5mM and 1mM respectively. NaCl inhibited the two activities, 50% inhibition of DNase and RNase occurring at 50mM and 10mM respectively. The activities were readily inactivated by heating, similar rates of inactivation being obtained for both. The properties of



the enzyme catalysing hydrolysis of denatured DNA and RNA are similar and the experiments summarised above provide further evidence that only one protein is responsible for the two activities.

8. The specificities of the two activities were studied. Both DNase and RNase were shown to have an endonucleolytic mode of action. Examination of their products of digestion on a column of DEAE-cellulose showed that only approximately 8% of the total digest consisted of mononucleotides. The remaining material contained dinucleotides and above.

9. The position of the terminal phosphate group of the oligonucleotides produced by the action of the enzyme was determined using snake venom and spleen phosphodiesterases. These experiments showed that the oligodeoxyribonucleotides and oligoribonucleotides were terminated by 5'-phosphate groups.

10. The specificity of the enzyme towards the bases was examined by determining the proportion of the bases at the 3' and 5' ends of the oligonucleotides produced by the digestion of DNA and RNA with the enzyme. These were determined by dephosphorylation of the oligonucleotides followed by digestion with snake venom or spleen phosphodiesterase. Treatment of the dephosphorylated



oligonucleotides with snake venom phosphodiesterase releases the base at the 5'-hydroxyl end as a nucleoside. Similarly digestion of the dephosphorylated oligonucleotides with spleen phosphodiesterase releases the base at the 3'-hydroxyl end as a nucleoside. These nucleosides were separated from mononucleotides, characterised and estimated. The experiments showed that the enzyme exhibited little specificity towards the bases in DNA and RNA at the point of attack.

11. The results of the study of the specificities of the enzyme towards DNA and RNA further illustrate the similarity of the DNase and RNase activities and suggest that a single protein is involved.

12. The possible functions of the nucleases identified in the liver are discussed.



**MINI COMPEY.**



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